THE BIOLOGICAL STANDARDISATION OF THE VITAMINS

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PREFACE TO THE SECOND EDITION

RECENT developments in the determination of the vitamins have made certain additions to and alterations in this book necessary. An International Standard of Reference for vitamin E has been adopted, and a sample of vitamin D_3 may soon replace the present International Standard for vitamin D, a sample of irradiated ergosterol in olive oil. It is now generally agreed that the limits of error of a determination should be calculated from the data supplied by that particular determination and not from a general estimate made from many determinations. Moreover, the development of methods of estimating fiducial limits of error to replace the old methods of estimating approximate limits through the interest and energy of such workers as Irwin, Bliss, Fieller, etc., has added something to the labour of calculating the limits of error though not as much as at first appears. I have made this part of the subject as simple as I can. The more mathematically minded readers will like to refer to the original papers. Again I would express my warm gratitude to Dr. J. O. Irwin for valuable help most generously given whenever I sought it.

My sincere thanks are tendered also to my publishers for the particular interest and help they have afforded in the production of both of the editions of this book.

KATHARINE H. COWARD.

BLOOMSBURY SQUARE,
LONDON, W.C.1.
February, 1947.

PREFACE TO THE FIRST EDITION

THIS book has been written in the hope that it may be of service to those workers who are engaged in the determination of the vitamin potency of foods, of special preparations for therapeutic purposes, or of products obtained in the course of investigations on the chemical nature of the vitamins. In giving practical details of experiments, I have drawn largely from my own experience, but I have been fully aware that in many laboratories the technique must be quite as good as my own, and more suitable to the local conditions than my own could be. I have, therefore, dwelt largely on the principles which should underlie biological

determinations of the vitamins, giving details of my own or others' technique only as I myself know them to be good. In particular I have stressed the need for International Standards of Reference and shown how they must be used so that a determination made in one laboratory may be reasonably concordant with one made in another laboratory, or in the same laboratory at a different time.

The book is divided into two parts. Part I deals with the practical side of the subject and may be used without reference to Part II if desired. Part II deals with the more mathematical treatment of the subject, the estimation of the accuracy of the results of experiments. It is a very elementary introduction to the study of statistical methods applied to vitamin determinations. It was written in the hope that even students who are not mathematically inclined will nevertheless read it and decide that the statistical treatment of results is really very simple after all. For students who wish to explore the possibilities further, there are available two excellent books, *The Methods of Statistics*, by Tippett (Williams and Norgate), and *Statistical Methods for Research Workers*, by Fisher (Oliver and Boyd).

No method of estimating vitamin E has been included since there is no international standard of reference for the factor yet.

I gladly take this opportunity of acknowledging my indebtedness to many people for training in various parts of my work, and of expressing my sincere thanks to them. To Professor J. C. Drummond I am indebted for six years of most generous teaching and guidance, not only in vitamin technique, but in the methods and aims of research generally; to Professor H. Steenbock for a very happy year of vigorous training during the tenure of a Rockefeller Travelling Fellowship at the University of Wisconsin; to Professor J. H. Burn for initiation into the principles of biological assay, and to Professor J. H. Gaddum and Dr. J. O. Irwin for many helpful discussions on statistical methods.

Acknowledgment is made to the Cambridge University Press and to the authors of the papers concerned for permission to reproduce Figs. 8, 9, 15, 17–21, 22, 26, 27, 35, 40, 42, 43 from the *Biochemical Journal*; to the Medical Research Council and His Majesty's Stationery Office for permission to reproduce Figs. 23, 31 from their Special Report Series No. 158; and to the Health Organisation of the League of Nations for permission to reprint the whole of Appendix I of this book from the *Quarterly Bulletin of the Health Organisation*.

KATHARINE H. COWARD.

LONDON,
November, 1937.

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PART I

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1. Standards of Reference

EVERY worker on the biological activity of the vitamins is familiar with the difference in response given by different animals to the same dose of any particular preparation of a vitamin, and it is evident that the greater the number of animals used for testing a dose of vitamin, the more nearly will the average response obtained approach the true average response to that dose. A much more serious difficulty than this has, however, to be faced. Not only do individual animals vary in their response to a given dose but the response of the whole stock of animals to a given dose has been shown by several workers to fluctuate gradually over long periods of time. This means that the potency of a preparation can never be measured simply by measuring the response of animals to a certain dose of it at any time, for six months later, or a year or five years later, the response of animals to the same dose would almost certainly be

greater or less than the original response. Moreover, it is obviously impossible to expect that the response of animals to a given dose in one laboratory would be equal to the response of animals to the same dose in another laboratory. To measure the potency of a substance simply by measuring the animal response would lead to widely different determinations and the greatest possible confusion.

The solution of this difficulty is easily available. For each of the vitamins A, B_1, C, D and E a standard of reference has been adopted for international use. A particular preparation of each vitamin was made and presented to the Health Organisation of the League of Nations, who asked the National Institute for Medical Research, London, to hold it and distribute it as required. The Institute continues to do this. The biological activity of a certain weight of each preparation is accepted as a unit of activity. Any worker desiring to use the standards may obtain them on application to the distributing agent in his own country. It is, however, of the utmost importance that each worker should understand how the standards must be used. The purpose of a standard of reference is to provide a measure, in terms of the unit adopted, of the biological activity of a substance. A dose of a substance which produced a response equal to that produced by, say, three units of the Standard would be said to contain three units of activity and the activity would be expressed in units per gramme. But since the response to a given dose of a vitamin fluctuates, it is absolutely essential that each test should be a comparison between the response given to a dose of the test substance and the response given to a dose of the Standard *at the same time.* In every test half the animals available should be given doses of the test substance and the other half should be given doses of the International Standard. Only in this way, comparison with the Standard by simultaneous testing, can the activity of a preparation be determined and stated in terms of the International unit. Certain workers are credited with having obtained a sample of a standard, tested a dose (or doses) of it on their own stock of animals, noted the response and never used it again; but any dose of material which at any subsequent time produced a response equal to that produced originally by a dose of Standard was declared to have a potency equal to that dose of Standard. These workers have completely ignored the fluctuations of their colony; indeed they

have probably never recognised them. Hence the whole value of the use of the Standard has been lost and erroneous determinations of vitamin potency have been made. It cannot therefore be stated too strongly that whenever a vitamin determination is required, simultaneous tests of the Standard and test substance must be made, and since the response of animals to a dose of Standard varies as much as the response to a dose of vitamin in any other form, equal numbers of animals must be used for the Standard and for the test substance.

When standards of reference for other vitamins become available, the same principles will govern their use as govern the use of the standards already adopted, for it is impossible to imagine an animal reaction that does not show fluctuations from time to time throughout the whole colony, or one that does not differ in different laboratories in which conditions of lighting, heating and diet must necessarily vary very much.

2. The Choice of a Criterion

The criterion chosen by the worker will be an animal reaction with which he is already familiar, but when the reaction is to be measured, the starting and end points have to be defined as carefully as possible. The life or death of an animal at a given time after receiving a dose is perhaps the most definite end point in a biological test, but few criteria have this degree of exactness ; *e.g.* the cure of xerophthalmia in rats requires a very exact description of the condition which shall be deemed the starting point for dosing. The condition which shall be deemed "cured" is still more difficult to define. Even "increase in weight" is not an exact measurement for one has to decide when a rat may be considered steady in weight and ready to receive doses. Also a rat's weight may vary by a few grams from hour to hour. Sometimes, as in the "line-test" or X-ray test for vitamin D, a scale has to be drawn up showing graded amounts af response appropriately marked or apprised, so that by comparison with the scale, the reactions of the rats in future tests can be marked or estimated, and the reactions of all the rats of a group thereby averaged. Sometimes several changes are involved in the criterion chosen as in the "guinea-pig's tooth" method for determining vitamin C. A table of changes is then drawn up and appropriate figures applied to the several collective stages.

3. The Curve of Response

The shape of the curve of response to graded doses of the vitamin must then be determined. Large groups of animals in as nearly as possible the same condition of weight, depletion of the vitamin, etc., should be given graded doses of the vitamin (preferably daily at first), each member of a group receiving the same dose. There should be equal numbers of male animals in all groups, and equal numbers of female animals, but it is not necessary to have the numbers of male and female animals in one group equal. If there are as many as, say, 40 animals in each group and nearly 20 of each sex, the results obtained can also be treated for males and females separately. If the two sexes give curves of the same slope, it will simplify the planning of future experiments greatly. It may be worth while at first to have a group in the experiment receiving no dose of the vitamin, though the results may not be used in the calculation of the slope. For example, if the curve obtained from the other groups proves to be logarithmic, the results from the "no dose" group cannot be used as there is no logarithm of 0. In many experiments the rats given no dose would die before the expiration of the experimental period, but if they did not, the group would form a useful comparison with the group given the lowest dose chosen. This lowest dose should be a very small one indeed, and the doses given to the other groups should each be double of the one immediately smaller than it. Few animal reactions will distinguish satisfactorily between one dose and another which is not at least double of that one, unless very large numbers of animals are used. The highest dose should be one which will give approximately the highest possible response. It is almost impossible, however, to get all the animals of a group to give a maximum response. It cannot be expected even in an "all or none" reaction. There is nearly always an "odd man" who behaves differently from the rest. The average results from the various groups are plotted against the doses given to find approximately the shape of the curve of response. If it appears to be logarithmic, the results are then plotted against the logs. of the doses given, when a straight line will be obtained if the original curve was indeed logarithmic.

One or two points will almost certainly not be on any straight line connecting or nearly connecting most of the points, and it

may be difficult to decide just where the best straight line through the points should be. There is, however, a mathematical procedure which determines the position of the best straight line through points which do not lie exactly on a straight line. It may be illustrated from the results of an experiment carried out in the writer's laboratory. The mean increases in weight in 3 weeks of five separate groups of male rats which had become steady in weight on a diet deficient in vitamin A and which had then been given doses of 0·25, 1·0, 1·5, 2·5 and 7·5mg. respectively of cod liver oil per rat per day, were —11·2, 7·3, 9·5, 16·8 and 33·4g. respectively. The curve of response relating the increase in weight in 3 weeks of rats so treated is found thus :

Increase in weight in 3 weeks, y.	Daily dose of cod liver oil, mg.	Log. of daily dose of cod liver oil, x.	Deviation from mean $\bar{x}$, $x-\bar{x}$.	Product of y into $x-\bar{x}$, $y(x-\bar{x})$.	Square of deviation from mean $(x-\bar{x})^2$.
G. 1 −11·2	0·25	$\bar{1}$·3979	−0·7715	+8·6408	0·5952
2 7·3	1·0	0·0000	−0·1694	−1·2366	0·0287
3 9·5	1·5	0·1761	+0·0067	+0·0636	0·0000
4 16·8	2·5	0·3979	+0·2285	+3·8388	0·0522
5 33·4	7·5	0·8751	+0·7057	+23·5704	0·4980
5)55·8		5)0·8470		34·8770	1·1741
11·16 $=\bar{y}$		0·1694 $=\bar{x}$			

$$b \text{ (the slope of the curve)} = \frac{\Sigma y(x-\bar{x})}{\Sigma (x-\bar{x})^2} = \frac{34{\cdot}8770}{1{\cdot}1741} = 29{\cdot}71.$$

The straight line is represented by the equation

$$\begin{aligned} y &= \bar{y} + b(x-\bar{x}) \\ &= 11{\cdot}16 + 29{\cdot}71(x - 0{\cdot}1694) \\ &= 6{\cdot}13 + 29{\cdot}71x \text{ (Fig. 1).} \end{aligned}$$

Hence the curve relating increase in weight and dose (mg.) of C.L.O. given is represented by the equation

$$y = 6{\cdot}13 + 29{\cdot}71 \log. x \text{ (Fig. 2).}$$

If the curve of response proves to be logarithmic, it may be assumed in future tests of the same kind that the response is logarithmic. Then two groups only of rats may be given appropriate doses of the International Standard (though some workers prefer three), and to get the curve of response, the average

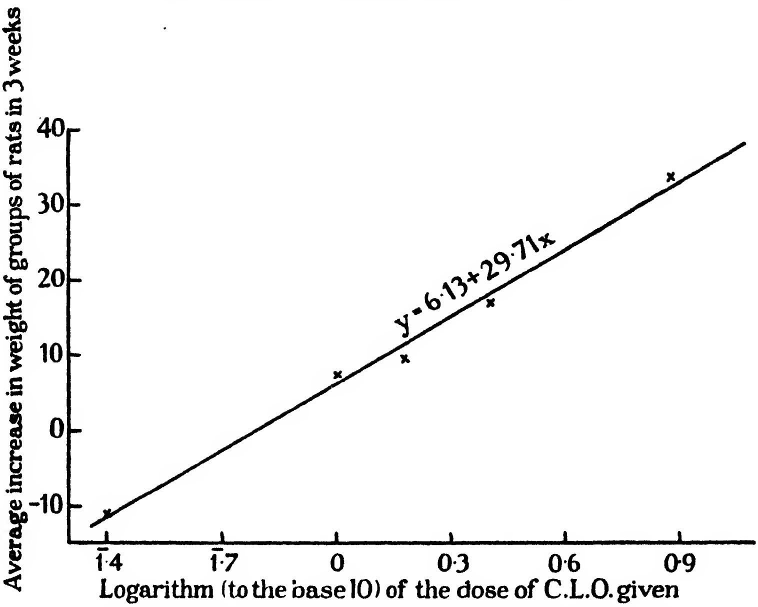

FIG. 1.—Curve of response relating increase in weight of male rats in 3 weeks and the logarithm (to the base 10) of the dose of cod liver oil given.

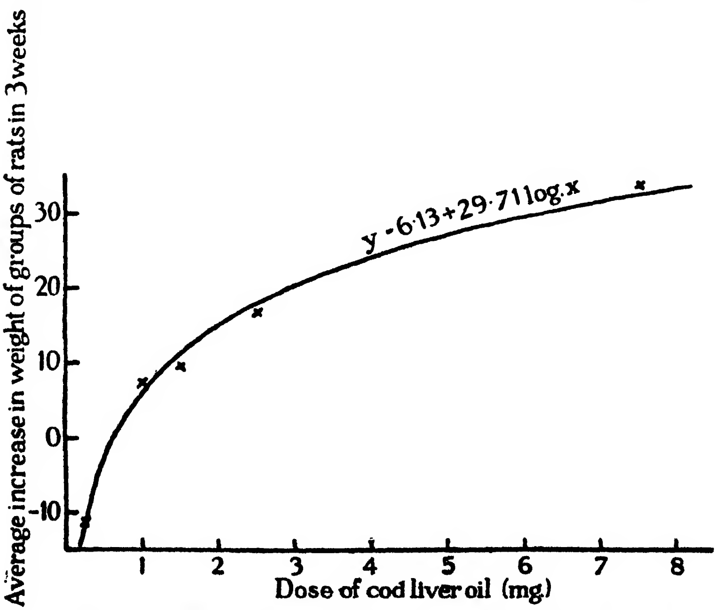

FIG. 2.—Curve of response relating increase in weight of male rats in 3 weeks and the dose (in mg.) of cod liver oil given daily.

results from the two groups may be plotted against the logs. of the doses given and the line joining the two points may be taken as the curve of response for these two groups. (In general, of course, it is desirable to have two groups of rats on the International Standard and two on the substance under test, determine the two curves and average the slopes, but this is dealt with more fully in the appropriate sections.) It is, perhaps, surprising how many curves of response are logarithmic in shape.

The curve of response may prove to be S-shaped, especially where the response to a dose is an "all or none" reaction, *e.g.* in the cure of retracted neck in a pigeon by a dose of vitamin B_1. To obtain the best curve through the points obtained experimentally, the "normal equivalent deviation" corresponding to the percentage of birds cured may be plotted against the log. of the dose given. Theoretically this gives a straight line, and the best straight line obtainable from the results of the experiment may be obtained by the same procedure as just outlined for the logarithmic curve, substituting the "normal equivalent deviation" found in Pearson's *Tables for Statisticians and Biometricians*, corresponding to the percentage of birds cured and using those fractions for y. For example, suppose a dose of

(*a*) 0·015g. International Standard produced 25% cures.
(*b*) 0·03g. International Standard produced 48% cures.
(*c*) 0·06g. International Standard produced 80% cures.

Then the curve of response is calculated as follows :

Percentage birds cured.	y.	Dose.	Log. of dose, x.	$x-\bar{x}$.	$y(x-\bar{x})$.	$(x-\bar{x})^2$.
25	−0·68	0·015	$\bar{2}$·1761	−0·3010	+0·20470	0·09061
48	−0·05	0·03	$\bar{2}$·4771	0	0	0
80	+0·84	0·06	$\bar{2}$·7782	+0·3010	+0·25287	0·09061
	3)0·11		3)$\bar{5}$·4314		Σ=0·45757	Σ=0·18122
	0·037		$\bar{x}=\bar{2}$·4771			

$$y=0{\cdot}037+2{\cdot}5(x-\bar{x})$$
$$=3{\cdot}8+2{\cdot}5x$$

$$b=\frac{0{\cdot}45757}{0{\cdot}18122}=2{\cdot}5$$

The slope of the curve is $b=2{\cdot}5$. See also Chapter XI.

The curve of response relating to dose of vitamin given may be a straight line but this seldom happens. When it does, and

the doses chosen have proved to be suitable, then the calculation of potency is simple, but the estimation of the accuracy of the test is more involved. An example of this kind of curve is seen in Key and Elphick's determination of vitamin C by the "guinea-pig's tooth" method (Chapter V).

In choosing a criterion for a biological assay the steepness of the curve of response should be considered, for the steeper the curve, the greater the accuracy obtainable. Moreover, male rats may give a steeper curve of response than females. This is so in vitamin A tests, and the difference between the slopes is more than enough to make up for the greater variation in the male rats' responses than that in the females'. Indeed the writer found that it required twice as many female as male rats to obtain the same degree of accuracy in vitamin A tests.

4. The Basal Diet

The basal diet of the animals used in a biological test must contain abundance of every food factor necessary for the health of the animal except the one vitamin under test. Otherwise, in a determination of, say, the vitamin B_1 content of a food substance, if the basal diet were not rich enough in one of the other B vitamins and this was supplied in the tested food, the response to the food would be enhanced and it would appear that the food contained more vitamin B_1 than it really did. The adequacy of the basal diet can be tested by constructing curves of response to graded doses of (*a*) the purest form of vitamin B_1 known and (*b*) some food substance such as dried yeast which contains other B vitamins. If the two curves have the same slope, or slopes not differing significantly, then the basal diet may be considered adequate. If they do differ significantly, the response to the food being steeper than the response to the pure vitamin, then the basal diet is not adequate. See Chapter VIII.

5. Statistical Examination of Results

Sir Henry Dale has said that "the object of every committee on biological standardisation is self-extermination." Chemical and physical determinations of vitamins are more quickly carried out than biological ones, and hence are preferable provided they are measuring the right thing. One of the strongest motives for making biological tests as accurate as possible is to obtain results

with which the results of chemical and physical tests may be compared. This involves estimating the accuracy of both the biological and the chemical or physical tests. Fortunately statistical methods of determining the accuracy of results are available and they have, in fact, been developed greatly in the last few years. It may be emphasised at this point that it is just as important to know whether two apparently equal results may really be considered equal, as it is to know whether two different results are *significantly* different. Nothing is really proved by statistical treatment of results. A conclusion is stated as a probability ; *e.g.* (*a*) two results are often stated to be significantly different if there is a one-in-twenty (or less) chance that they are in fact equal, or (*b*) the potency of a substance may be given as x units per gram, with limits of error 67 to 150% for $P=0{\cdot}95$, which means that in 100 determinations of the potency of that substance 5 out of the 100 results (approximately) would be outside 67 to 150%, or, stated otherwise, there is a one-in-twenty chance that the result obtained is either less than 67 or more than 150% of the true result. Similarly limits of error for $P=0{\cdot}99$ are the limits outside which one result in a hundred would lie, or there is a one-in-a-hundred chance that the result obtained is in fact outside these limits. A statement of potency giving limits of error without mentioning probability means absolutely nothing. The British Pharmacopœia Commission has given the limits of error usually obtained for biological tests in terms of $P=0{\cdot}95$ and $P=0{\cdot}99$. The estimates of these limits were based on very large numbers of experiments from various laboratories, but it was realised that many determinations would show a greater or less degree of accuracy than this when examined from their own figures only. In fact, the growing trend of opinion is that each experiment should be judged on its own merits, which means that the slope of the curve of response must be determined in each experiment. This is most fruitfully done by dividing the animals available into four (or six) groups and averaging the slopes obtained from the two (or three) groups used for the standard and the two (or three) groups used for the substance under test. The difference in slope between the two curves as well as the distance apart both contribute to the error of the test.

An example of a comparison between biological and chemical and physical determinations of vitamin B_1 was reported by the

vitamin B Sub-committee of the Accessory Food Factors Committee. Certain samples of flour and bread made from the flour were examined by (*a*) the thiochrome method with visual and spectrophotometric comparison, (*b*) the azo method and (*c*) biological methods (increase in weight in rats, cure of convulsions and bradycardia). Good agreement was obtained by all these methods for the flour. The chemical test gave a significantly lower content of vitamin B_1 in the bread than in the flour, which suggested a loss in baking. The biological test also indicated a lower value for the bread, but this was covered by the error of the test, and so there might in fact have been no loss in the baking. One is left wondering whether the chemical method did not extract and estimate all the vitamin from the bread while it had probably done so from the flour, and also, whether the rats had done so from both.

6. Planning an Experiment

The accuracy of a biological determination can be much increased by careful planning of the experiment which can also simplify the calculation of the result and the estimation of the accuracy obtained. After carrying out many determinations by the same method and under conditions as nearly identical as they can be made, the worker will have gained some ideas as to what factors may influence the response of an animal to a given dose of a vitamin, *e.g.* body-weight when first given the basal diet, or bodyweight when first given the dose of vitamin. Working out a correlation coefficient will indicate whether such a correlation really exists, though if it apparently does not, it is worth while working it out again after more data have been accumulated, for the first set of figures may not be large enough to indicate the correlation.

When evidence of some such correlation has been formed, it will be considered when arranging rats in groups for further experiments.

Different groups of animals should contain equal numbers of rats, and where sex has been shown to influence response, the numbers of male rats should be the same in all groups, and likewise the numbers of females, but it is not necessary to have the same number of males and females within a group.

"Litter-mate" control is very desirable indeed, especially in

vitamin D determinations where all animals are given the same length of preparatory period without regard (or only very little) to the degree of rickets developed. If it is possible to reduce animals approximately to the same degree of deficiency which is indicated by some criterion, such as cessation of growth or maintenance or loss of weight as in vitamin A determinations, then perhaps litter-mate control is of less importance, but even under these conditions Irwin has lately shown that litter-mate control reduces variation in vitamin A assays. If no information is available concerning the probable potency of the substance to be tested, a preliminary experiment should be performed with doses very widely separated in order to obtain some idea of the doses that should be used in the main experiment.

After a few years' experience with a stock of animals some idea of its vagaries and gradual fluctuations in various ways will have been gained. The worker will have realised that much valuable information on the biological reactions of animals may be obtained from routine assays. Most of the material from which this book has been compiled was obtained while working out and applying methods for the routine analysis of food samples. The reader is recommended to read Fisher's *Design of Experiments*, and even if the more difficult parts are omitted or postponed for a future reading, a wider view and appreciation of the possibilities of experimentation will undoubtedly be gained.

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CHAPTER II

ANIMALS SUITABLE FOR THE DETERMINATION OF VITAMINS BY BIOLOGICAL METHODS

RATS are used for the determination of vitamins A, B_1, D_2, D_3 and E, and chicks for the determination of vitamin D_3 only. Pigeons and sometimes rice birds are used for the determination of vitamin B_1, and guinea-pigs are used for the determination of vitamin C. All workers on vitamins are agreed that it is essential to control the breeding and feeding of rats which are to be used for vitamin tests and that this can best be done in a room given up to this purpose in the building where the tests are to be carried out. It is not necessary to control the breeding and feeding of pigeons before using them for tests, nor is this necessary for guinea-pigs provided that the animals are obtained from a clean and healthy stock.

1. Rats

A. The housing of the rats.

Daylight is not necessary for the breeding of rats; thus a room without windows can be usefully employed in housing the rat colony. If the room available for the rats has windows which do not face north, they should be covered with blinds. This is to prevent irradiation of the rats at irregular intervals with ultra-violet rays which might make their reserves of vitamin D vary too much. If the room faces north, the use of blinds is probably unnecessary.

The whole of the interior of the room should be washable, and the floor should slope down to a drain so that it may be easily cleaned. There should be as little woodwork in the room as possible; one bench or table may be allowed, but it should be made of teak or mono-metal with metal supports. A good supply

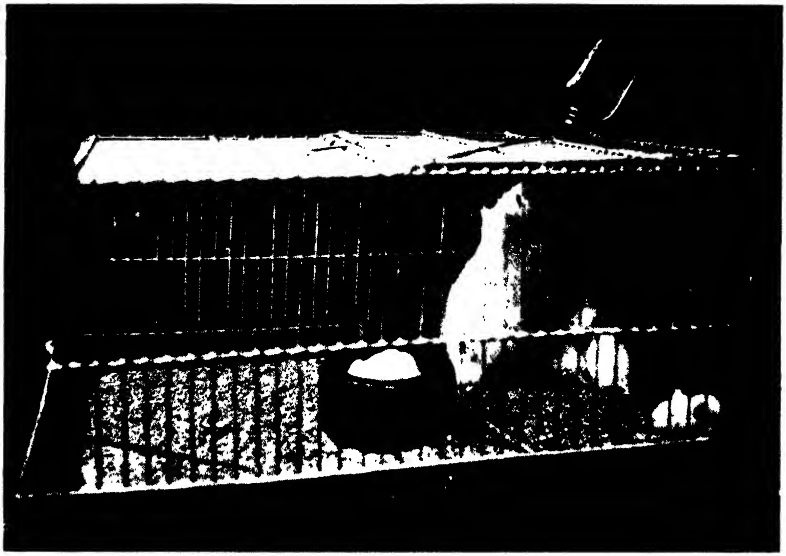

FIG. 3.—A useful type of rat cage.

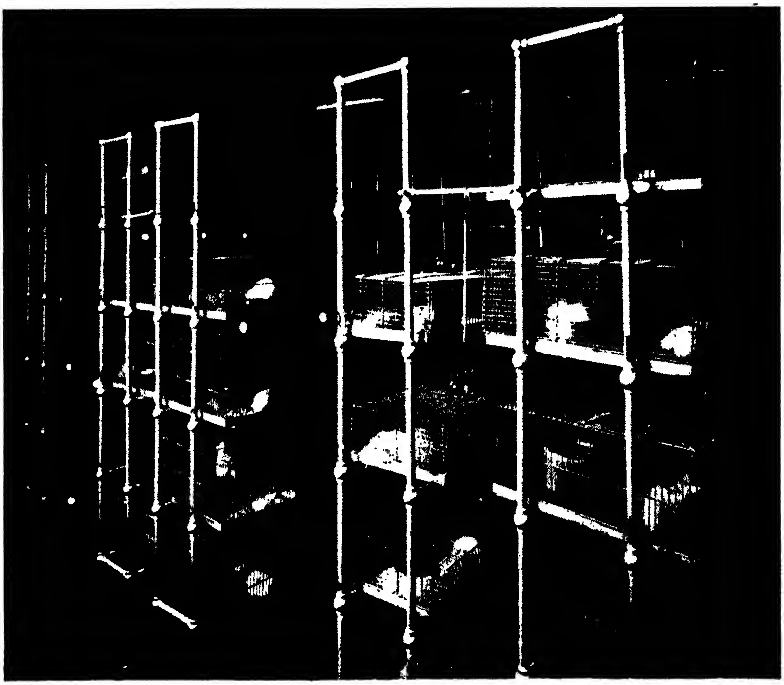

FIG. 4.—Simple fitting for a rat room.

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of hot and cold water is necessary and a large sink and draining board are useful. All cage-cleaning is done in another room.

The room should be kept at a fairly constant temperature of about 65–70° F., though this is not so necessary for the rat colony as it is for the experimental rats, many of which must be considered sick animals. Ventilation is necessary both for the rats and for the workers. Draughts, of course, are undesirable. Adequate artificial lighting is essential.

It is necessary to keep the rats in cages which are large enough to allow them a fair amount of movement, but the cages must be not too heavy to be moved easily. One simple form consists of a galvanised iron tray, 3″ deep with a loose upper part (sides and top only) made of wire, in some form of open-work, about 8″ high, standing in the tray. The sides of the tray provide protection from draughts and prevent the scattering of the bedding, while the open-work top allows access of fresh air. The top should have a hinged door, either in the top to open upwards or in the front to open forwards. It should be large enough to allow the worker to get a hand and arm inside the cage for catching a rat or changing a food-pot. The "overall" size for such a cage is about 12″ (long) × 18″ (deep) × 8″ (high) (Fig. 3).

The stands for these cages can easily be made of hollow gas-tubing, jointed to any measurement desired. There should be enough space between the tiers to allow the cages to be pulled in and out easily and to allow a water-bottle (described below) to rest on top. Thus about 12″ should be allowed between tiers. Stands may be arranged against the walls of the animal room, and, if space allows, back to back down the middle (Fig. 4). Enough space should be left somewhere in the room to allow for changing of cages; that is the transferring of the rats from a dirty cage to a clean cage which is most easily done on a fairly low table.

There are many types of water-bottles is use in different laboratories; some are simple and some are elaborate. The essential point is that the rat should have clean water every day and that it should be supplied in an inverted vessel so that it cannot be fouled. A simple form is an ordinary glass bottle with a neck large enough to allow the inside to be easily cleaned occasionally and provided with a straight delivery tube wide enough at one end to fit into the neck of the bottle with a piece

of rubber tubing, and drawn out at the other end to a diameter small enough to hold in the water when the bottle is inverted, and yet large enough to let the rat get the water easily by licking it. The tube of the water-bottle is fixed between the wires of the upper part of the cage.

Bedding should be supplied liberally. It may consist of saw-dust or wood-shavings, preferably of pine-wood or deal. It has been stated that shavings of teak are harmful to rats.

When several rats are kept in one cage of the size mentioned above, the cages should be changed at least twice a week. Where a doe has a cage to herself awaiting parturition, and during the lactation of her young, it is enough to change her cage once a week. Dirty cages should be removed from the rat room, scraped, washed with soapy water and rinsed well in clean water. They may then be stacked to dry overnight. In a healthy colony sterilisation of the cages is not necessary. If occasional rats are suspected of being unhealthy, their cages should be washed in 5% lysol and well rinsed in clean water, for lysol is toxic to rats as well as to bacteria.

Food-pots should have inturned upper edges to prevent the rat from scratching out the food on to the floor of the cage. They should be washed thoroughly at least once a week.

Many elaborate arrangements have been designed for the housing and feeding of experimental animals, but in most places it is possible to find suitable structures and utensils made locally which are very much cheaper than many which are obtainable elsewhere. The cost of running a rat colony is necessarily high but sound economy can be practised by using material which is easily available.

B. **The diet of the rat colony.**

It must not be assumed that a diet found to be satisfactory for the growth and reproduction of rats in one laboratory will necessarily be equally satisfactory in another laboratory. The diet must be good enough for growth and reproduction but must be low enough in vitamin content to ensure low reserves of these factors in the young. To strike a balance between these two needs is sometimes very difficult for it is not possible yet to bring up a colony on a purely synthetic diet. All diets contain some substances such as yellow maize, wheat, dried milk or fresh milk and even casein which must vary in composition. The sample

of any one of these obtainable in one part of the country may be different from the sample obtainable in another and the difference may be just great enough to make a diet known to be satisfactory in the one part, unsatisfactory in the other. Moreover, it is to be expected that a diet which has proved satisfactory even for a year or two in one place may need some modification from time to time. It is very unlikely that the average reserves of any factor in a stock of rats will remain stationary over a long period of time, even with a diet kept as nearly uniform as possible. A diet which the writer (Coward, Cambden and Lee, 1932) has found satisfactory for some sixteen years consists of :

Yellow maize, ground finely	65·0 parts
Whole wheat, ground finely	20·0 ,,
Caseinogen, light white (B.D.H.)	9·0 ,,
Dried brewer's yeast	5·0 ,,
Sodium chloride	0·5 ,,
Calcium carbonate	0·5 ,,
Dried milk	20·0 ,,
Iron citrate *	0·1 ,,
Potassium iodide *	0·1 ,,

In addition to this mixture, which is supplied to the rats *ad lib.*, some green food (watercress, cabbage, or even grass cuttings) is given in amount about 4g. per rat per week and also fresh uncooked liver or beef, about 4g. per rat per week. An extra 5% of dried yeast is added to the diet for the lactating does.

It is an interesting but unexplained fact that although the diet has supported a vigorous colony of albino rats for some twenty generations, yet three different attempts to establish a colony of black and white rats on this diet have failed, even though the young piebald rats had been obtained from three different sources.

A diet devised by Steenbock (1923) and found satisfactory through many generations of rats in his Wisconsin laboratories has the following composition :

Yellow corn, ground finely	76·0%
Linseed oil meal	16·0%
Crude casein	5·0%
Ground alfalfa	2·0%
Sodium chloride	0·5%
Calcium carbonate	0·5%

In addition to this, the rats received a generous supply of fresh cow's milk, brought straight to the laboratory from the

* Additions made since the publication of the paper.

University farm and given to the rats within 2 hours of milking. Certain cows belonging to the herd of the University were always fed on a winter ration to keep the vitamin D potency of the milk low. The diet for the young does was supplemented with 5% butter.

However, when the writer adopted this recipe for the diet for her colony at its beginning in 1927, the rats in the second generation failed to grow normally and though they produced some litters, they did not wean them. Even when the diet was supplemented with extra vitamins, A, B complex, D or E or A+D, no improvement was found and it was concluded that the diet, as made in the London laboratory, was lacking in some unknown factor which was supplied in the diet as made in the Wisconsin laboratory. It seemed very probable that it was the milk (the ordinary London pasteurised supply) which was inferior to the fresh milk of the Wisconsin supply. This has never been proved by experiment though later work in this laboratory and in others lends weight to the probability. Kon (1931) who had worked in the Wisconsin laboratories and knew the value of the Steenbock stock ration, found that the ingredients available in his laboratory at the State School of Hygiene, Warsaw, did not make an adequate diet; nor was he able to get good results with diets compounded according to the formula of Professor Evans of California University.

Again, Smith and Anderson (1929) reported failure of a diet based on the formula that Sherman and Campbell (1924) had found adequate for many generations of rats, their only modification of the Sherman diet being the addition of calcium carbonate (1% of the wheat constituent), of fresh lettuce *ad lib.*, and, for the lactating does, 9g. of tested dried yeast per week. It is impossible to believe that any of these additions lowered the value of the ration and yet it did not produce such good results as the Sherman diet in the Columbia laboratories.

All of these failures are open to the suggestion that it was not the diet which was at fault but some other item in the management of the colony. Coward again put her form of the Steenbock diet to the test, however, when she had her own stock diet giving good results, so that the only difference in treatment of the two lots of rats was in their respective diets. Again the Steenbock diet, as made with materials available in London, failed.

Thus there is abundant evidence that a diet which has been found completely satisfactory in one laboratory may not be found satisfactory in another when made with materials that are pardonably thought to be the same but which in reality may be very different. Each worker who has a colony of rats must work out for himself a suitable diet from the materials available in his laboratory. Until this is done, it is advisable to make careful records of the breeding of the individual rats, and compare them with records of other workers. Otherwise, it is only too easy to think that a colony is behaving badly or well when actually it may be the reverse.

C. **The strain of rat suitable for vitamin tests.**

The domesticated form of the rat, *Mus norvegicus,* is suitable for experimental purposes. In particular the albino rat, *Mus norvegicus albinus,* is bred by many investigators, though others breed the piebald variety.

The albino rat has been bred for experimental work for a great many years at the Wistar Institute, Philadelphia. Greenman and Duhring (1931) have written a full account of the procedure there.

Inbreeding of carefully selected rats is advantageous for the production of a uniform strain. Brother-sister mating in a healthy stock seems to have no unfavourable results (King, 1918), but it is often inconvenient to keep strictly to this method and it is certainly unnecessary for vitamin studies.

The rats may be mated when the does are about 150g. weight, and the bucks somewhat heavier. Does can produce and lactate their young when mated at a lower weight than 150g. but their milk supply may then be insufficient for the litter and the first mating may be wasted. The ingestation period is normally 21–22 days. Lactation should be carried on for 21–25 days though the young can sometimes be weaned at 14 days if needed. A doe can produce and rear 5–6 litters with an average of 5–6 rats per litter when fed on a diet such as is described above. It is often advisable to allow a doe to rest for two weeks between the weaning of a litter and the next mating. These figures were obtained from the records of Coward, Cambden and Lee (1932) for their colony of albino rats fed on the diet described above which was somewhat restricted in quality to ensure low reserves of the vitamins in the young. Many rat breeders whose only aim has been to produce large numbers of healthy young rats

have evidence of much more prolific breeding than this, but their diet is probably richer than any of those described in this chapter.

It is of great value to keep full records of the breeding of a colony, at least until one is satisfied with the diet which has been adopted for it. The rate of growth of the rat should be determined from birth to the first mating by weighing weekly, then averaging bucks and does separately, and drawing composite growth curves from the averages. It may be of interest for some purposes to note the age and weight of each doe when her vagina opens. It is certainly worth while to examine vaginal smears daily in order to ascertain whether the stock is having œstrous cycles regularly. This is done by inserting a small

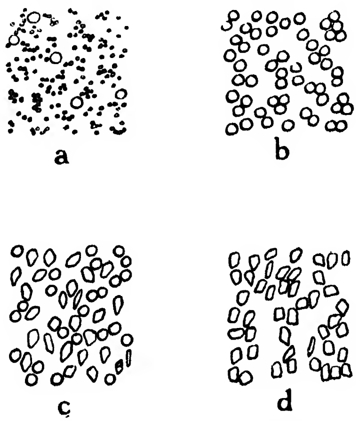

FIG. 5.—The contents of the vagina during the œstrous cycle.

spatula into the vagina of the rat, pressing the edge against the vaginal wall and withdrawing a little of the contents. This is rubbed off on to a drop of water on a slide and examined under the low power of a microscope without staining. The œstrous cycle in the rat has been fully investigated by Long and Evans (1922). They find it to recur every 5 or 6 days in the normal animal and to show the following stages:

a. Diœstrus, numerous leucocytes and a variable proportion of nucleated cells for about 3 days.

b. The beginning of œstrus—no leucocytes, numerous nucleated cells—for about 6 hours.

c. Œstrus, the best time for mating—nucleated cells and keratinised epithelial cells for about 24 hours.

d. The ending of œstrus—numerous keratinised cells for about 24 hours (Fig. 5).

The cycles are often irregular in unhealthy rats. Dietary deficiencies may be detected by irregularities in the cycles before they are detected in any other way. A shortage of vitamin A in the diet leads to the presence of keratinised cells in the vagina daily, whereas a shortage of vitamin B (the complex) (Evans and Bishop, 1922) or of vitamin B_1 (Coward, Morgan and Waller, 1942) leads to a complete absence of keratinised cells.

The best time for mating a doe is when the vaginal contents consist of a mixture of nucleated and keratinised cells, or of keratinised cells only. The doe will not receive a buck at any other time. When copulation has taken place, spermatozoa are generally found in the vaginal contents the next morning. A plug may or may not be found loosely filling the lower end of the vagina. From a healthy buck, the spermatozoa are very numerous and often found in large sheafs. They may even be still motile when examined. From about the 12th to the 16th day after a successful copulation there is a certain amount of bleeding into the vagina, and the smear consists of a little clot of blood, quite easily recognised by the naked eye. If this is absent no litter will be born. There are no cycles during ingestation. Records of matings, the occurrence or absence of spermatozoa and plug in the vagina, the occurrence of blood clots in the vagina, and the ingestation period should all be recorded for each doe. Loss of weight after the occurrence of a blood clot in the vagina and subsequent failure to produce a litter indicates that resorption after implantation of fertilised ova has taken place. It may be due to a lack of vitamin E in the diet.

Coward, Cambden and Lee (1932) recorded the ingestation period of 358 litters together with the success or failure of the does in lactating these litters, during the time when the Steenbock diet (as made of materials available in their laboratory) was slowly proving itself inadequate. The colony was obviously lacking in some dietary essential, and it was shown that the longer the ingestation period the smaller was the percentage of litters weaned (Fig. 6).

Thus by keeping full records of the growth and reproduction of the rats of a colony, some information may be obtained as to possible deficiencies before such deficiencies would otherwise become apparent. Even if no deficiencies are detected, it will have been well worth the time expended on making the observations

to be sure that the colony is behaving in a normal way. Variations must be expected. The behaviour of the rats of a healthy colony with regard to growth and reproduction are no more uniform than any other animal reaction, and a knowledge of their

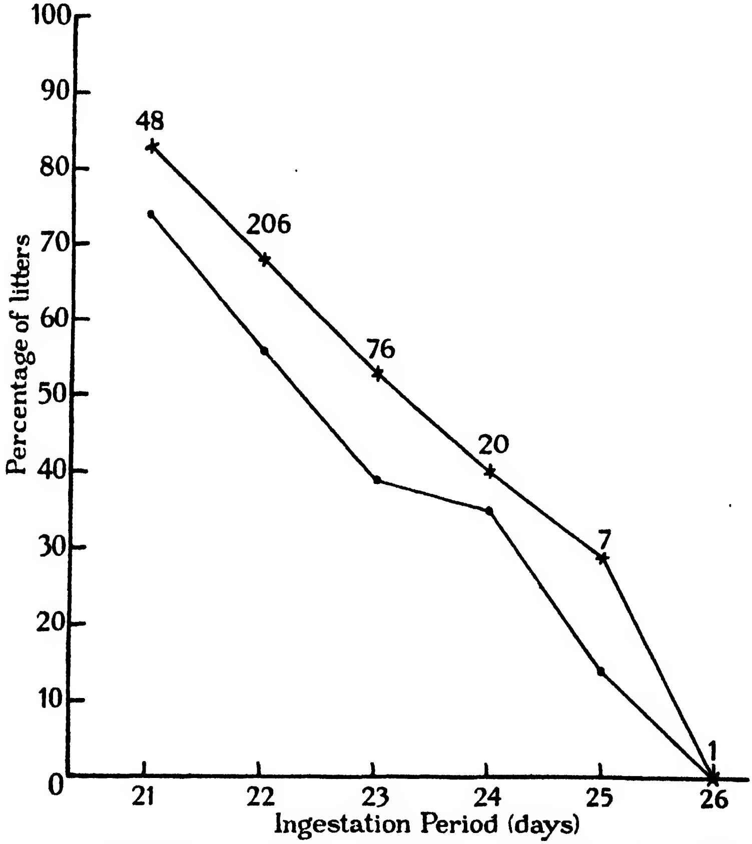

FIG. 6.—The relation between the mortality of young rats and the length of their ingestation period.

Number at each point indicates number of litters born.

★ = percentage that lived one week.

● = percentage that were weaned.

variation is exceedingly useful for comparison with the variation encountered in all vitamin experiments.

D. Seasonal fluctuation in the fertility of rats.

For many years during which records have been kept in the writer's laboratory of the numbers of does mated and numbers

of litters born month by month throughout each year, a serious decline in the percentage of litters born during November, December and January has been noted. Various suggestions for correcting this have been made by other breeders of rats and tried out in the writer's colony, but without complete success. For example, one worker thought he had kept up the breeding during the winter months by giving the rats chopped bone with bone marrow. When that was tried in the writer's colony the rats became so wild and savage that it was difficult to handle them. After a month the treatment was discarded without having produced any appreciable effect on the breeding. Another worker claims to have kept up the fertility of his rats during the winter months by a very lavish use of artificial light (ordinary electric light bulbs). It does not seem to the writer, however, that lack of light can be the cause of the slowing down, for improvement in the numbers of litters weaned is noticeable at the end of January which means that those litters were born at the beginning of January and copulation must have taken place three weeks before that, the whole of this six weeks period being at the time of least daylight. Some improvement in the percentage of litters weaned was, however, gained by omitting the fortnight's rest between weaning a litter and remating the doe, but in no year of the colony's 16 years of otherwise smooth running has fertility in the last two months of the year equalled that in the rest.

2. Guinea-pigs and Pigeons

Since vitamin C is made by the rat, that animal cannot be used for vitamin C determinations. Guinea-pigs are used for this work, and since vitamin C is not stored to any appreciable extent in the guinea-pig's body, it is not necessary to limit the amount of this factor in the food of the stock of guinea-pigs for work on vitamin C. Guinea-pigs may be obtained from any reliable, healthy stock as needed, kept for two or three days in the laboratory to recover from possible disturbances of a journey and then used for experiment at once.

The same procedure may be adopted for pigeons which are to be used for experiments on vitamin B_1 and also for rats which are to be used for this purpose, since neither rats nor pigeons store appreciable amounts of this factor.

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CHAPTER III

THE DETERMINATION OF VITAMIN A

1. The International Standard of Reference and Unit of Vitamin A Activity

In 1931 the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations recommended that carotene be adopted as a provisional International Standard of reference for vitamin A. Seven workers in different laboratories each prepared 4–5g. of carotene which were sent to the National Institute for Medical Research, London, where they were pooled, recrystallised and distributed in ampoules of 10mg. each. This preparation served as a standard of reference until further knowledge of the chemistry of the lipochromes revealed the fact that this was not a pure substance but a mixture of substances. In 1934, therefore, the Permanent Commission on Biological Standardisation recommended the substitution of pure β-carotene for the International Standard of reference and asked Professor Karrer to prepare the necessary sample. This was

done and the preparation presented to the League of Nations. It was sent to the National Institute for Medical Research, London, where its distribution was undertaken by the Department of Biological Standards. It has the following properties:

(*a*) Melting point, 184–184·5° C.
(*b*) Optically inactive.
(*c*) Absorption spectrum showing bands: in carbon disulphide at 519 and 485·5mμ, in chloroform at 495 and 465mμ, in cyclohexane at 486 and 456mμ.

The preparation actually sent out to workers is a solution of the sample of β-carotene in coconut oil of a concentration of 500 units per gram; *i.e.* 300μg. of β-carotene per gram.

The unit of vitamin A activity is defined as that amount of activity contained in 0·6μg. of the International Standard of β-carotene. This is the amount contained in 2mg. of the solution as issued by the National Institute for Medical Research. It is equal in biological activity to 1μg. of the preparation of carotene provisionally adopted in 1931. The unit of the later preparation therefore is equal in biological value to the unit of the earlier preparation, though it has lately been suggested that it is not really so (Hume, 1943). The weight of β-carotene, 0·6μg., was chosen so that the biological value of the unit should not change. Hence a preparation which was found to contain, say, 1,000 International units of vitamin A activity when tested against the provisional Standard, should also be found to contain (within the limits of experimental error) 1,000 International units when tested against the new Standard.

A. **The dilution of the Standard of reference for dosing.**

Since a daily dose of two or three units of vitamin A is sufficient to bring about a response in rats as they are usually prepared for the test, the International Standard of reference is too concentrated to use as issued. The oil to be used for its dilution must be chosen with great care. Some oils slowly destroy β-carotene as shown by a loss of colour of the solution. Obviously such oils are unsuitable for dilution of the Standard. The International Standard is made up in coconut oil and, for its dilution, a sample of coconut oil containing 0·01% of hydroquinone should be used which has recently been shown to have no destructive effect on β-carotene dissolved in it. This may

be determined by matching the depth of yellow colour of a weak solution (say, 0·003%) against the yellow (and red) glasses of a tintometer or against a 0·5% solution of potassium bichromate in a colorimeter, before and after storage at 37° C. for seven days; if the loss of colour is not more than 10% the coconut oil may be considered satisfactory as a diluent of the Standard solution of carotene.

A summary of the evidence on which the recommendation was based by the authority issuing the International Standard was made by Hume and Chick in the Special Report No. 202 of the Medical Research Council. It was suggested in this report also that the greater activity of β-carotene dissolved in coconut oil than of β-carotene dissolved in some other oils (*e.g.* one sample of arachis oil, one sample of olive oil) might indicate a more complete utilisation by the rat of β-carotene from this solvent than from others, but Coward (1936), from somewhat indirect evidence, has shown that this is probably not the explanation of the apparently lower activity of β-carotene in some of the oils than in others.

The possibility of the vitamin A activity of cod liver oil being enhanced or diminished when the oil is diluted with other oils for dosing should also be recognised. An early result of the writer indicated a greater activity of vitamin A when the cod liver oil was diluted with coconut oil than when diluted with olive oil; but a recent comparison with different samples of coconut oil, olive oil and arachis oil has given very concordant results (unpublished results). With 19–20 rats in each of three groups, the rats of the different groups being given a daily dose of 1mg. of a particular sample of cod liver oil dissolved in coconut oil, olive oil and arachis oil respectively, the potency of the cod liver oil in the different solvents was found to be in the ratio 0·98 : 1·35 : 1·30, from which it may be concluded that these particular samples of coconut, olive and arachis oils had the same influence on the vitamin A of the cod liver oil.

Suggestions for the use of the Standard are sent out with the first batch to every worker.

B. Need for a simultaneous test on the Standard of reference whenever a vitamin A determination is made.

In making a biological test for any vitamin, it is of the greatest importance that a simultaneous test be made on the Standard

of reference. Not only do individual animals vary in response to a given dose of any vitamin, but the whole of the rats of a colony may show gradual fluctuations in their response to the same dose of a vitamin, generally without a recognisable cause.

This may be illustrated for vitamin A by figures obtained in the writer's laboratory in successive tests on the International Standard of reference, β-carotene. (Table I).

TABLE I

VARIATIONS IN RESPONSE (AVERAGE INCREASE IN WEIGHT) OF GROUPS OF ANIMALS TO THE SAME DOSE, 1 OR 2 UNITS OF THE INTERNATIONAL STANDARD FOR VITAMIN A (β-CAROTENE).

	1 I.U.				2 I.U.			
	♂		♀		♂		♀	
	n	I	n	I	n	I	n	I
December, 1944 *					6	14·7	8	10·4
September, 1944 *	4	5·2	9	3·3				
May, 1943 *	6	−16·2	11	−0·2	5	−4·0	12	10·1
March, 1943 *	7	7·4	12	3·7	7	13·1	13	12·1
December, 1942	4	2·3	13	6·2	4	17·3	11	15·5
November, 1942	4	4·5	12	15·5	4	2·25	11	17·4
June 1942	7	9·1	11	5·5	7	18·4	13	16·4
November, 1941					8	21·4	8	18·4
May 1941	8	−4·9	6	7·8				
September, 1940	7	8·6	5	9·6				
July, 1940	2	0	8	10·0				
June, 1940	4	3·25	6	8·7				
May, 1940	4	3·75	7	6·3				
May, 1940	2	13·0	8	4·0				
April, 1940	6	10·8	7	11·0	6	21·7	9	20·7
March. 1940	6	3.8	7	2·3				
February, 1940	5	4·2	7	4·4				
February, 1940	2	−1	8	9·75				
November, 1939	6	20·7	6	16·8				

* In these tests each rat was given 1mg. α-tocopheryl acetate in 20mg. of arachis oil once a week.

n = number of animals in the group.

I = average increase in weight in 3 weeks of the group.

The results amply demonstrate the need for making a simultaneous test on a standard of reference whenever a substance is to be tested for vitamin A. Other results indicate a similar need of a standard of reference for each of the other vitamins.

One point in regard to the use of a standard of reference in all biological tests may be mentioned here. It is essential to use about the same number of animals in the test on the Standard as in the test on the substance whose vitamin potency is to be determined. That is, the same degree of accuracy is needed in determining the influence of the Standard at the time of the test as in determining the influence of the unknown substance. When "litter-mate" comparisons are being made, it is obvious that each litter should be represented equally in all groups of animals.

C. **The general arrangement of the test for the determination of the vitamin A content of a substance in terms of the International Standard.**

There are two ways of arranging a test for determining the vitamin A content of a substance in terms of the International Standard. The rats may be given a vitamin A-free diet until their reserves of vitamin A are exhausted and they show signs of vitamin A deficiency. They are then given daily or twice a week doses of the test substance or of the Standard of reference for a specified period of time. On the other hand, they may be given the vitamin A-free diet plus doses of the test substance or Standard from the beginning of the experiment. In the first method, often spoken of as the curative or therapeutic method, the response to the vitamin is measured by the animals' behaviour from the day they were given the first dose of test substance or Standard. In the second method, often spoken of as the preventative or prophylactic method, the response to the vitamin is measured by the animals' behaviour from the beginning of the experiment. Each method has advantages over the other method which will be discussed later.

Whichever method is used, equal numbers of animals should be used for testing the Standard and the substance under examination. When increase in weight is the criterion adopted for the comparison, the proportion of male rats to female rats should be the same in the two groups, though the number of male rats need not equal the number of female rats. If this arrangement cannot be made, an adjustment of the calculation of the result can rectify it.

The rats to be used for testing the Standard are divided into two groups as nearly alike as possible, and the rats for the substance under examination are divided similarly. The two groups

for the Standard are then given doses of the Standard in the ratio 2 : 1 and those for the substance under examination are given doses in the ratio 2 : 1 also if some information of its possible potency has already been gained, but in the ratio 3 : 1 or 5 : 1 if no such information is available. The object of the experiment is to find a dose or doses of the substance under test comparable with a dose or doses of the Standard. This arrangement of the test can be applied to both the curative and the prophylactic methods, but in the latter method a group of "no dose" rats should be included and the test carried on for a few weeks after the "no dose" rats have died.

In order to average the response of different animals to the same dose, some method of measuring the response must be adopted. For the "increase in weight" method, the weights of the different animals are easily averaged. For the cure of ophthalmia the numbers of days taken to effect a cure are averaged, but the condition of the eyes that may be considered the starting point for the "curative period" and the condition when they may be considered cured must be clearly recognised by the worker concerned. It is not necessary that workers in different laboratories should adopt exactly similar conditions as their criteria. Comparison with the Standard of reference by a simultaneous test makes that unnecessary.

2. Preparation of Rats for Vitamin A Determinations

Certain arrangements for vitamin A tests can be made whichever criterion of vitamin A depletion is to be used.

A. **Animals suitable for the test.**

It is essential to have rats for vitamin A tests whose reserves of vitamin A are small, otherwise they may grow to maturity on a vitamin A-free diet. In a curative test, they would not give a starting point for the test and in a prophylactic test they would not show any difference in effect between the vitamin A-free diet and that plus a dose of the unknown or of the Standard. The diet of the stock colony must therefore be regulated accordingly. Details of a suitable diet for a colony which is intended to supply rats for vitamin A tests have been given in Chapter II.

B. **Housing of the animals.**

The room in which experimental animals are kept should be reasonably large and airy. Overcrowding should be avoided, partly for the sake of the rats and partly for the sake of the workers in the rat room. Several medium-sized rooms are preferable to one large one for various obvious reasons. The usual considerations of cleanliness apply to experimental animals as well as to the colony. The temperature of the room should be kept as nearly constant as possible at 20° C. A cold night may have disastrous results on the rats of a vitamin A experiment.

The cages of animals which are to hold a whole litter of 8–10 rats during the depletion period should be about 12″ long, 18″ deep (back to front) and 8″ high, and made of galvanised iron. The base should be solid with a rim 3″ high standing up all round it. The top part should be quite separate from the base and made of wire.

During the test period when each rat is given a dose of test substance or of Standard, it should have a separate cage of dimensions at least 12″×9″×6″ (high).

Bedding may consist of shavings or sawdust. Wire screens to raise the rats from the sawdust are not necessary in experiments on vitamin A, though some workers find it convenient to use wire mesh screens with sheets of blotting paper or newspaper in the tray. These are absorbent and easily renewed every day.

A north aspect is probably the best for a room in which vitamin A tests are to be carried out, but it is quite possible that direct sunlight would be without effect on rats fed on a diet deficient in vitamin A but well supplied with vitamin D.

C. **Vitamin A-free diet (basal diet).**

The vitamin A-free diet must be as nearly as possible free from vitamin A, and must not vary in this respect though the use of the Standard of reference will to a great extent control variations in results due to unavoidable and slight variations in the vitamin A content of the basal diet. Moreover, the diet must contain an abundance of all other factors known to be necessary for the well-being of the experimental animal. This point should be proved to each worker's complete satisfaction before any experiments are begun, and also at intervals later whenever any serious change is made in the stock of any ingredient of the diet. It may easily be done by giving the diet to,

say, two or three litters of young rats, until their reserves of vitamin A are exhausted and then giving each one a large supplement of the purest form of vitamin A available. Growth should be resumed at a rate equal to that of the stock colony. If not, the diet should be investigated before any further experiments are undertaken. It was an experience of this kind that led Coward, Key and Morgan (1929) to the detection of what was probably a previously unrecognised vitamin.

A diet which has been found suitable for vitamin A tests in several laboratories consists of:

Caseinogen	15%
Dextrinised rice starch	73%
Dried brewer's yeast	8%
Salt mixture (Steenbock's 40)	4%

This is supplemented by giving each rat 8–10 International units of vitamin D per week. A solution of irradiated ergosterol or of calciferol of known strength is diluted so that one drop of the solution, 0·02g., contains 8–10 units of vitamin D. One drop of the solution can conveniently be given directly into each rat's mouth once a week.

Since Moore (1940) showed that vitamin E influences the storage of vitamin A in the liver of rats, the precaution has been taken of giving a dose of 1mg. α-tocopheryl acetate in 20mg. arachis oil to each rat weekly during the preparatory and test periods. No evidence of any advantage of this procedure has been observed in this laboratory and indeed Bacharach (1940), putting the matter to carefully controlled experiment, failed to confirm it. Evans, however, as long ago as 1928 showed that vitamin E had growth-promoting properties and this was confirmed by Emerson and Evans (1937) and by Olcott and Mattill (1937). It should therefore be included in the diet of rats used for vitamin A determinations. (See also Chapter VIII.)

The kind of caseinogen used in this diet is of great importance. Most samples of caseinogen probably contain a trace of vitamin A which is carried down with it from the milk from which it is prepared. Some workers remove most of the fat and vitamin A by extraction with alcohol and ether, but this is a laborious process and very costly; other workers heat the caseinogen in thin layers in an oven at 105° C. for 24 hours, stirring it occasionally to expose fresh surfaces to the air and thus destroy, by oxidation, the traces of vitamin A.

Four samples of caseinogen have been found suitable for vitamin A tests by the writer :

1. "Light white" casein, sold by the British Drug Houses Ltd., but not prepared by them. Information as to the method of preparation has been refused by the makers.

2. A sample of caseinogen kindly prepared by the British Drug Houses Ltd., by the text-book method of precipitation by dilute hydrochloric acid from diluted skimmed milk.

3. A sample of caseinogen also kindly prepared by the British Drug Houses Ltd., by precipitation with alcohol.

4. Various samples of sodium caseinate, "Physiological caseinate," prepared for the market for vitamin tests by the Glaxo Laboratories Ltd.

The casein, which for some reason still undetermined was found to be unsuitable for vitamin test, was the kind previously sold by the Glaxo Laboratories Ltd. as vitamin-free casein. It is certain that some workers found this form of casein satisfactory. It is equally certain that other workers besides the writer did not. In view of the fact that many animals were able to make full use of it as a protein, it does not seem to be probable that it was a biologically poor protein. The possibility is suggested that "light white" casein contained, as an impurity, some substance (which may be another vitamin) in which the Glaxo vitamin-free casein was lacking ; and that the rats which grew well on the Glaxo casein did so because they held reserves of the substance on which they could draw, whereas the rats which did not grow well had no reserves. A full account of the work has already appeared in the literature and has received support from Mapson (1932, 1933) who has been able to make an extract of liver which apparently makes good the deficiency of the Glaxo vitamin-free casein. It indicates that this may be a purer, not a poorer, form of casein than the others which have been found satisfactory. Whatever may prove to be the explanation, the practical outcome of the work is to emphasise the necessity of ensuring that the basal diet for a vitamin test should contain abundance of all factors necessary for the well-being of the animal except the one factor for which the test is being made. This can only be determined by supplementing the diet with the purest form available of the missing vitamin to see whether "normal" growth can be induced in rats whose reserves of that particular vitamin have been exhausted.

Almost any kind of starch is suitable for a vitamin A-free diet,

but if the diet is used in the dry state, the starch, being very finely powdered, collects in the rats' fur, which is unsatisfactory. Moreover, raw corn starch which is freely used in vitamin work in America, is not easily digested by rats. Also, rats which have eaten uncooked starch, particularly potato starch, have been found to be subject to a condition known as refection in which undigested starch is excreted in the fæces. For all these reasons, it is desirable to cook the starch somewhat. Some workers mix the starch with enough water to make it stand in solid lumps and then autoclave it. Others mix the starch with enough water to make a thin paste, then boil it to burst the starch grains, then spread the paste on flat tins and bake it over hot pipes until brittle. A simple method found satisfactory by the writer is to mix the starch with enough water to make solid lumps, break these into small pieces and bake them in flat baking-tins in an ordinary gas cooking-stove until brittle. Whichever method of cooking is adopted the partially dextrinised starch must be ground to a fairly fine powder for mixing in the diet. It will keep in this state almost indefinitely.

The dried yeast should be tested for vitamin B (complex) potency, sample by sample, from any one source until it has been shown to be of reasonably constant potency. It must be ensured that the quantity incorporated in the diet allows for "normal" growth when abundance of vitamin A is also supplied.

Various salt mixtures have been used by different workers with equal success. Steenbock's mixture, No. 40 (Steenbock and Nelson, 1923), reproduces as nearly as possible the salt content of cow's milk. It consists of :

Sodium chloride ($NaCl$)	23·36 parts
Magnesium sulphate ($MgSO_4.7H_2O$)	24·6 „
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	35·8 „
Dipotassium hydrogen phosphate (K_2HPO_4) ..	69·6 „
Calcium phosphate ($Ca_2H_2(PO_4)_2.4H_2O$)	68·8 „
Calcium lactate $.5H_2O$	15·4 „
Iron citrate $.6H_2O$	5·98 „
Potassium iodide (KI)	0·16 „

Osborne and Mendel's salt mixture (Osborne and Mendel, 1919) which many workers have used, consists of :

$CaCO_3$	134·8g.	Citric acid $+H_2O$ ..	111·1g.
$MgCO_3$	24·2g.	Fe citrate $1\frac{1}{2}H_2O$ ..	6·34g.
Na_2CO_3	34·2g.	KI	0·02g.
K_2CO_3	141·3g.	$MnSO_4$	0·079g.
H_3PO_4	103·2g.	NaF	0·248g.
HCl	53·4g.	$K_2Al_2(SO_4)_4$ ..	0·0245g.
H_2SO_4	9·2g.		

The chemicals used were analysed and allowance was made for moisture, etc. The acids were mixed and the carbonates and ferric citrate added to them. The traces of KI, $MnSO_4$ and $K_2Al_2(SO_4)_4$ were added as solutions of known concentrations. The final resulting mixture was evaporated to dryness in a current of air at 90–100° C. and ground to a fine powder.

One salt mixture (185) used by McCollum, Simmonds and Pitz (1916) which has also been used by other workers consists of:

NaCl	0·173g.	
$MgSO_4$ (anhydrous) ..	0·266g.	
$NaH_2PO_4.H_2O$	0·347g.	
K_2HPO_4..	0·954g.	
$CaH_4(PO_4)_2.H_2O$ ..	0·540g.	
Ca lactate	1·300g.	(Iron citrate 1·0g. was added by some workers.)

A comparison of these three shows how very varied may be the salt mixture given to experimental animals, all of these different mixtures being apparently equally good.

A generous supply of fresh water should be given. Tap water from the laboratory is generally suitable. There is no need to use distilled water.

3. Criteria for the Measurement of the Response of Rats to Doses of Vitamin A

It is recognised by all workers on vitamins that vitamin A is stored in the animal body in varying amounts, and that when the animal is fed on a diet deficient in vitamin A its reserves of that factor are gradually exhausted, and certain symptoms appear in the animal, some of which are apparently typical of vitamin A deficiency. If the animal continues to be deprived of vitamin A the condition becomes more severe, and in some 10–20 days from the first appearance of the symptoms the animal dies. Post-mortem examination reveals various lesions which were not recognisable in the living animal, *e.g.* a large proportion of rats which die from a deficiency of vitamin A are found to have abscesses at the base of the tongue, or they may have congestion of the lungs or various intestinal disorders. These conditions, however, are not obvious in the living animal and, indeed, are not specific for vitamin A deficiency. The symptoms which are useful in experiments on the determination of vitamins are those which can be recognised in the living animal before the condition has become too severe to be cured, unless the criterion

to be measured is simply the survival period after the giving of a dose of vitamin A.

The most useful criteria for determinations of vitamin A in food substances are :

(*a*) Increase in weight of young animals.
(*b*) Xerophthalmia.
(*c*) Irregularities in the desquamosa of the vagina of the rat.

When young rats whose reserves of vitamin A are not very great are given a diet deficient in that factor, they continue to put on weight for some time but eventually cease ; they may develop a painful condition of the eyelids and later of the eyes (though curiously this does not happen in some laboratories) and in female rats, after the vagina has opened, a smear of the contents of the vagina is found to contain keratinised epithelial cells continuously day after day, instead of at the normal intervals of about five days.

The difficulties of making any of these measurements are obvious. A rat does not suddenly cease to grow on a particular day nor is the onset of xerophthalmia a sudden occurrence ; several days must pass before it is certain that a rat's weight has become steady, or that the condition of the eyelids has reached the required degree of severity. There must be therefore some uncertainty as to the exact day to terminate a prophylactic experiment or to begin a curative one. A similar difficulty is met again towards the end of the curative test. Even in measuring increase in weight the fluctuations in weight of an animal throughout any day prevent the measure from being as accurate as it might be expected to be. The vaginal smear method appears to offer a sharp "end-point," but it is not always a simple matter to decide whether the keratinised epithelial cells can be considered really to have disappeared from the vaginal contents, for a few stray ones sometimes occur during the diœstrous period of normal rats. Also since keratinised epithelial cells appear for 2 days during the normal œstrous cycle, if the normal occurrence is followed by, or preceded by, the abnormal occurrence of keratinised epithelial cells day after day, there might easily be an error of two days in the measurement. This particular difficulty could be overcome by ovariectomising the rats, when œstrous cycles would cease and the normal occurrence of keratinised epithelial cells in the vagina would cease also.

The difficulties of determining "end-points" by any of the criteria so far described appear to indicate that the death of the animal, the only certain end-point, must be the best criterion of all. No work on this method has been published, and it might produce good results, but probably the length of time required to wait for the death of all animals would make the method cumbersome and unpractical.

A. Increase in weight as the criterion for the determination of vitamin A.

α. THE "CURATIVE" METHOD

(*a*) *The preparatory period.*—Young rats weighing as little as 25–30g. may on weaning be given the vitamin A-deficient diet. If they do not grow at all, rather older rats should be used. The best rats for the test are those that become steady in weight on a vitamin A-deficient diet at about 80–90g. They should be weighed once a week for the first 3 weeks of the preparatory period, then twice a week until they begin to grow more slowly, then every day or two when they cease to put on weight. After this they decline in weight rapidly. Thus it is important to weigh them frequently in order to recognise the real steadying in weight. Until this happens all the rats of a litter may be kept in one cage, but as each rat becomes steady in weight it should be given a separate cage for the rest of the experiment.

The number of rats to be prepared is determined from the considerations outlined on pp. 27, 28, 181. A few extra ones should be allowed as occasionally rats die through being left too long on the preparatory period.

Some workers insist on having isogenic pairs of rats, *i.e.* pairs of rats of the same sex from the same litter, one of each pair for the test on the Standard and one for the test on the substance under examination. In a prophylactic test this is, of course, essential and also in a test in which the reserves of vitamin A are only partly exhausted when dosing is first begun. But the writer has calculated (unpublished results) from large numbers of test animals in her laboratory that when the rats' reserves of vitamin A are exhausted and they become steady in weight, no greater accuracy is obtained from the use of isogenic pairs of animals than from rats of different litters. In her laboratory for several years a continuous supply of young rats has been prepared on a

vitamin A-free diet and each day the rats which are considered to have become steady in weight are assigned in turn to the groups intended for the testing of (*a*) the Standard of reference, (*b*) the substance under examination, and often (*c*) another substance under examination. It appears to be of greater importance to start the test period of equal numbers of rats in the different groups on the same day than to wait a few days for "litter mates" to become steady for the test. Tourtellotte and Bacon (1935) have shown that temperature affects the response of rats to vitamin D; it is easily conceivable that it affects the response to vitamin A and that other conditions may affect it also. It is essential, then, that equal numbers of rats receiving the Standard and the test substance shall be subject to the same variations of environment, and the only way to ensure this is to assign equal numbers of rats to the different groups each day until the required number is obtained. Irwin, however, has found from data supplied by another worker that greater accuracy was obtained with "litter mates" than with rats selected by the same workers as described above.

(*b*) *The test period.*—Dosing with the test substance and Standard respectively are continued for the same length of time, the rats being weighed once a week during this period. The length of the test period must depend on the desired accuracy of the result. The period used for a long time was 5 weeks, but the writer has shown that a result is only slightly less accurate if the test period is shortened to 4 or even 3 weeks (Coward, 1933). In 2 weeks only, the inaccuracy of the result is much greater and no period less than 3 weeks can be recommended. In order to get the final weight at the end of the test period as accurately as possible, it is useful to weigh the rat on three successive days, the 20th, 21st and 22nd of the test and then take the average of the three weighings as the final result.

(*c*) *Dosing the rats.*—Until a few years ago doses of the Standard and of the test substance were given daily, but Coward and Key (1934) showed that the same result was obtained if the same total amount of Standard or of test substance was given in two doses per week only. The variation in response to this way of dosing was not greater than that in the daily dosing. (The values for σ for the male rats were 13·03 and 11·96 respectively, and for the female rats 9·45 and 9·81 respectively for the two ways of dosing.)

This method of dosing is convenient only when the substance tested is of such high potency that an amount equal to 3 or 4 days' doses can be given on one day. Less potent preparations should be given daily. Substances of very low potency may be incorporated as a percentage of the basal diet, but if this is done records of food consumed daily must be made, *e.g.* 25g. of the substance to be tested may be finely powdered and mixed with 100g. of the basal diet, *i.e.* it is incorporated in the diet as 20% of it. A weighed amount of this (say, 10g.) is given to each rat, in a feeding pot that cannot be upset and that is large enough to prevent the rat from scratching out the food from it, each rat being housed in a separate cage. The amount not eaten should be weighed the next morning and made up to the previous weight by a fresh addition. Remnants not eaten should be thrown away only if moist or seriously soiled, for it is always possible that, in a dry mixture, some part of the food, possibly the test substance, may have sunk to the bottom of the pot and so have not been eaten. Taking out the remnants to weigh them and mixing them with a fresh portion of food gives the rat a second chance of eating it. It is advisable when a substance has to be tested in this way to give a rat only about 2g. in excess of what he is likely to eat from day to day, and also to make only 125g. of the mixture (100g. basal diet+25g. test substance) at a time and to use all of that before using a second small batch. Each day the bulk should be well stirred to mix again evenly before use. It is even advisable to keep a separate batch for each rat to ensure his having received, finally, the full amount of test substance that the records of his food consumed indicate.

The daily or half-weekly dose of a liver oil or of the International Standard, both suitably diluted with the same oil, may be given as one drop from a small dropping tube or pipette. Short glass tubes 3″ long and ¼″ internal diameter fitted at one end to a narrower tube are suitable for the purpose. The size of the drop of a particular solution depends on the external diameter of the tube. A convenient practice is to make two or three dozen of these and then select for use only those which deliver, say, not more than 20·5mg. and not less than 19·5mg. of the solution. The dose is then given by letting fall one drop (or more if required) of the solution directly into the rat's mouth from the tube held upright. Greater accuracy can be attained

by the use of a micrometer syringe of the Agla type fitted with a blunt injection needle. The end of this is held in the rat's mouth by one worker while the other turns the screw until the required amount has been delivered. This method of dosing is strongly recommended by the writer who ceased using dropping tubes many years ago.

Sometimes the substance to be tested is a solid and so cannot be dropped into the rat's mouth. The weighed portion can generally be given to the rat by putting it, a small portion at a time, into his mouth. A drop of water or inactive oil is given after each mouthful. Generally a rat will quickly swallow everything put into its mouth, but it should be put on to a flat clean table for a minute and watched to make sure that it has done so.

(*d*) *Working out the result.*

Example (i).—Suppose the following figures have been obtained in a 3-weeks' test:

6 rats given 0·5mg. C.L.O. daily made a mean increase in weight of 2·0g.
6 rats given 1·0mg. C.L.O. daily made a mean increase in weight of 9·3g.
6 rats given 2·0mg. C.L.O. daily made a mean increase in weight of 23·4g.
6 rats given 0·5 unit Standard daily made a mean increase in weight of 7·5g.
6 rats given 1·0 unit Standard daily made a mean increase in weight of 12·2g.
6 rats given 2·0 units Standard daily made a mean increase in weight of 28·0g.

The determination of the potency of the cod liver oil could be made thus:

Each dose of the cod liver oil has produced a smaller response than the corresponding dose of the Standard. Therefore the oil contains less than 1,000 International units per gram.

Also 1·0mg. of the C.L.O. has produced a greater response than 0·5 unit of the Standard and 2mg. of the C.L.O. have produced a greater response than 1·0 unit of the Standard. Therefore, the oil contains more than 500 International units per gram. It appears to contain about 800 units per gram.

The figures used for this example are more regular than are often found when small numbers of animals (*e.g.* 6) are used in a group. Even when less regular results are obtained, com-

parisons drawn in this way between doses of oil and doses of Standard may be balanced so that a very fair idea of the value of the oil may be obtained. Indeed, the value estimated in this way is probably as near the true result as that obtained by a more accurate calculation. The reader is recommended to work out the result by the method used in *Example* (iii), though the calculation will be somewhat simpler as there will be no weighting for numbers of rats used. It can be assumed that the rats were all of the same sex.

Example (ii).—A simple calculation of a result. The results from the male and female rats are calculated separately and averaged according to the number of rats which contributed each result. Since the response of rats to doses of vitamin A has been shown to be logarithmic, a straight line is drawn between the average increases in weight from the two doses of Standard plotted against the logs. of the doses given, and its equation is determined. A similar curve is drawn for the two points obtained from the substance under test. The slope of the curve is the value of b, *i.e.* the coefficient of x in the equation. The average slope of the two curves is the average of the two b's. It is most unlikely that they will be equal and they may be very different. (In the simplest arrangement, the number of rats in all four groups is the same. If not, certain modifications, which will be described later, have to be made.) Then the middle point of each curve is determined, *i.e.* the point with the average values for x and y; curves are drawn with the average slope through these "mid-points" and equations for them calculated. The horizontal distance between these parallel curves (straight lines, of course) is the log. of the ratio of the potencies of the corresponding doses of the Standard and the substance under test. Another figure for the relative potencies is obtained similarly from the responses of the female rats. The potency of the test substances is found by averaging the two results.

Suppose that:

10 rats(♂'s) gave an average increase in weight of 5·6g. from a dose of 1 International unit.

10 rats (♂'s) gave an average increase in weight of 14·5g. from a dose of 2 International units.

10 rats (♂'s) gave an average increase in weight of 5·1g. from a dose of 0·05g. vitamin margarine.

10 rats (♂'s) gave an average increase in weight of 19·2g. from a dose of 0·1g. vitamin margarine.

Calculate the result thus :

y increase in weight.	Dose.	x log. dose.	No. of rats.
5·6g.	1 I.U.	0	10
14·5	2 I.U.	0·3010	10
Diff. : 8·9		Diff. : 0·301	
5·1	0·05g. M.	$\bar{2}$·6990	10
19·2	0·1	$\bar{1}$·0000	10
Diff. : 14·1		Diff. : 0·301	

$$\text{Average slope is } \frac{8·9+14·1}{2\times 0·301}=38·2=b.$$

Point with average abscissa and ordinate on Standard curve is 0·151, 10·05. Point with average abscissa and ordinate on margarine curve is $\bar{2}$·850, 12·15. The curve with average slope, 38·2, through the "mid-point" of the curve for the Standard is

$$\frac{y-10·05}{x-0·151}=38·2 \qquad i.e.\ y=38·2x+4·28$$

The curve with average slope, 38·2, through the "mid-point" of the curve for the margarine is

$$\frac{y-12·15}{x-\bar{2}·850}=38·2 \qquad i.e.\ y=38·2x+56·08$$

The vertical distance between these lines is 56·08—4·28=51·80. The horizontal distance between these lines is

$$\frac{y}{x}=b$$

i.e.

$$\frac{51·80}{x}=38·2$$

therefore

$$x=\frac{51·80}{38·2}=1·3560$$

1·3560 is the log. of 22·699.

Therefore the potency of the margarine (in gram units) is 22·699 times the potency of the Standard (in International units).

Therefore the potency of the margarine according to the male rats in the test is 22·7 International units per gram. It is preferable to call this 23 International units per gram.

A similar calculation is made from the responses of the female rats used in the test and if the numbers of female rats was the

same as the number of males used, then a simple average of the results from males and females gives the final result for the potency of the margarine. It is preferable, really, to determine the accuracy of the results from the males and females separately and weight the mean according to the accuracy of each result. This is discussed in Part II.

Suppose, however, in this example each group of female rats had contained only 8 animals and the result obtained from them had been 18·1 International units per gram. Then the average of the two results from male and female rats respectively would have to be "weighted" according to the number of rats; that is, we should multiply the result from 40 males by 40, and that from 32 females by 32 (or by 5 and 4 respectively), add the products and divide by 72 (or 9) which would give an answer of

$$\begin{aligned} 22{\cdot}7\times 5 &= 113{\cdot}5 \\ 18{\cdot}1\times 4 &= \ \ 72{\cdot}4 \\ \hline &\ 185{\cdot}9\div 9 = 20{\cdot}66, \text{ say 21 I.U. per gram.} \end{aligned}$$

The simple average of 22·7 and 18·1 is 20·4 and we should have called the result 20 International units per gram. A figure in the decimals in this result would imply greater accuracy than the method justifies.

A short cut may be taken in this calculation by calling 0·05g. of margarine 1 part, and 0·1g. 2 parts. Then the abscissæ of the "mid-points" are the same, 0·151, and the equations with average slope through the "mid-points" of the two parallel curves are

$$\frac{y-10{\cdot}05}{x-0{\cdot}151}=38{\cdot}2 \qquad i.e. \quad y=38{\cdot}2x+4{\cdot}28$$

and

$$\frac{y-12{\cdot}15}{x-0{\cdot}151}=38{\cdot}2 \qquad i.e. \quad y=38{\cdot}2x+6{\cdot}38$$

The horizontal distance between the two parallel lines is

$$\frac{6{\cdot}38-4{\cdot}28}{38{\cdot}2}=0{\cdot}0550=\log.\ 1{\cdot}135$$

Then the potency of 0·05g. margarine is 1·135 times 1 International unit=1·135 I.U., and the potency of the margarine is 22·7 I.U. per gram, say 23 I.U. per gram.

Example (iii). A more complicated calculation: unequal numbers of rats in the different groups and two doses of Standard

and three of test substance given. (Result of an experiment in the writer's laboratory.)

Standard. Male rats.

y increase in weight, g.	Dose of Standard solution, g.	x log. dose.	n no. of rats.
25·9	0·002	$\bar{3}$·301	12
39·5	0·004	$\bar{3}$·602	13
Diff.: 13·6		Diff.: 0·301	

The slope b of the curve is $\frac{13 \cdot 6}{0 \cdot 301} = 45 \cdot 18$.

The "mid-point" is not exactly in the middle here because 12 rats were given the lower dose and 13 the higher. The difference 0·301 between $\bar{3}$·301 and $\bar{3}$·602 is therefore divided in the ratio 13 : 12 and the "mid-point" is nearer the higher value. Similarly the difference, 13·6, between 39·5 and 25·9 is also divided in the ratio 13 : 12 and the "mid-point" is nearer the higher value. The "mid-point" or point with average (weighted) values for x and y is therefore $\bar{3}$·458, 32·97.

Butter. Male rats.—To obtain the best straight line through the three points, relating increase in weight to dose of butter given, proceed as described in Chapter I but modify according to the number of rats in each group.

y increase in weight, g.	n number of rats.	ny.	Dose, g.	x log. dose.	nx.	$x-\bar{x}$.	$ny(x-\bar{x})$.	$(x-\bar{x})^2$.	$n(x-\bar{x})^2$.
16·8	12	201·6	0·025	$\bar{2}$·398	−19·224	−0·301	−60·6816	0·09060	1·08720
30·5	11	335·5	0·05	$\bar{2}$·699	−14·311	0	0	0	0
46·6	12	559·2	0·1	$\bar{1}$·0	−12	+0·301	168·3192	0·09060	1·08720
	35	35)1096·3			35)−45·535		107·6376		2·17440
		$\bar{y}$ = 31·32			−1·301				
					$\bar{x}$ = $\bar{2}$·699				

The slope b is $\frac{107 \cdot 6376}{2 \cdot 1744} = 49 \cdot 50$.

The "mid-point," that is the point with average values for x and y, is $\bar{2}$·699, 31·32 (already found).

The average slope for Standard and butter is weighted according to the number of rats used for each:

$$\frac{45 \cdot 18 \times 25 + 49 \cdot 50 \times 35}{60} = 47 \cdot 70$$

The curve with average slope, 47·70, through the "mid-point" of the curve for the Standard is

$$\frac{y-32\cdot97}{x-\bar{3}\cdot458}=47\cdot70 \qquad i.e. \quad y=47\cdot70x+154\cdot22$$

The curve with average slope, 47·70, through the "mid-point" of the curve for the butter is

$$\frac{y-31\cdot32}{x-\bar{2}\cdot699}=47\cdot70 \qquad i.e. \quad y=47\cdot70x+93\cdot38$$

The horizontal distance between these curves is

$$\frac{93\cdot38-154\cdot22}{47\cdot70}=-1\cdot2755$$
$$=\bar{2}\cdot7245$$
$$=\log. 0\cdot05303$$

Therefore the butter has 0·053 of the potency of the Standard solution (500 I.U. per g.).

Therefore it contains 0·053 × 500 I.U. per gram = 26·5 I.U. per gram.

The result obtained from the female rats used in the assay of this sample of butter was 35·63 I.U. per gram. Fifty-six female rats were used but the accuracy of the test proved to be less than that of the result obtained by the male rats, and when the average of the two results were weighted according to the accuracy of each the result was 29·8 I.U. per gram. For the method of weighting according to the accuracy of the test see Part II.

An alternative calculation of the average slope is made by the use of the formula

$$\frac{\Sigma n_1y_1(x_1-\bar{x}_1)+\Sigma n_2y_2(x_2-\bar{x}_2)}{\Sigma n_1(x_1-\bar{x}_1)^2+\Sigma n_2(x_2-\bar{x}_2)^2}$$

where $\Sigma n_1y_1(x_1-\bar{x}_1)$ is the sum of all the individual $y(x-\bar{x})$ on the substance tested

$\Sigma n_2y_2(x_2-\bar{x}_2)$ is the sum of all the individual $y(x-\bar{x})$ on the International Standard

$\Sigma n_1(x_1-\bar{x}_1)^2$ is the sum of all the individual $(x-\bar{x})^2$ on the substance tested

and $\Sigma n_2(x_2-\bar{x}_2)^2$ is the sum of all the individual $(x-\bar{x})^2$ on the International Standard.

In the last calculation, $\Sigma n_1y_1(x_1-\bar{x}_1)=107\cdot6376$

$\Sigma n_1(x_1-\bar{x}_1)^2 = 2\cdot1744$

$\left.\begin{array}{l}\Sigma n_1y_2(x_2-\bar{x}_2)\\ \Sigma n_2(x_2-\bar{x}_2)^2\end{array}\right\}$ would have to be calculated.

If two substances have been tested against the Standard at the same time, the average of the three slopes is obtained from the formula

$$\frac{\Sigma n_1 y_1(x_1-\bar{x}_1)+\Sigma n_2 y_2(x_2-\bar{x}_2)+\Sigma n_3 y_3(x_3-\bar{x}_3)}{\Sigma n_1(x_1-\bar{x}_1)^2+\Sigma n_2(x_2-\bar{x}_2)^2+\Sigma n_3(x_3-\bar{x}_3)^2}$$

β. The "Preventive" Method

In this method the rats are given doses of test substance or of Standard from the beginning of the experiment, though some workers give their rats a preparatory period of 2 (or even more) weeks during which no supplement is given to any of the rats. Thus the rats' reserves of vitamin A are not exhausted when the doses are first given and the success of the experiment depends on getting different average increases in weight in the two groups of rats given different doses of the Standard and in the two groups of rats given different doses of the test substance.

(*a*) *The test period.*—It is advisable to have five groups of not less than 10 rats in each group for a prophylactic test. Each group must contain the same number of rats, one or two, from each litter used, so that each litter is represented equally in each group. This is important as there is no treatment for reducing all the rats to a similar state of vitamin depletion as there is when a preparatory period of feeding on the vitamin-deficient diet is allowed. The rats may be 20–50g. in weight according to their reserves of vitamin A which can only be inferred from a general knowledge of the behaviour of the rats of the colony when given a vitamin A-free diet.

The doses of the Standard should be in the ratio 2 : 1 and the doses of the test substance also, if some information of the potency of the substance is already available. If not, then a preliminary test is made with doses in a greater ratio, say, 5 : 1, or, if preferred, several groups of rats may be given doses of the test substance in the ratio 8 : 4 : 2 : 1.

The doses are given in the same way as in a "curative" test, each rat being kept in a separate cage for the whole of the period during which doses are given. One week's dose may be given in two half-weekly doses instead of one-seventh of the amount daily. The rats are weighed once a week. The test is carried on until the control rats given no supplement have died and until there are distinct differences in the increases in weight of

the groups of rats given graded doses of both the test substance and the Standard.

All the rats should be kept on test for the same length of time, even if the different litters have been started at different times.

(*b*) *Working out the result.*—A "composite curve of growth" should be constructed for each group of rats. To do this, the average weights of the rats of each group at the beginning of the experiment and at the end of each week afterwards are calculated. The averages so obtained are plotted against the time and the slopes of the curves so constructed may be compared. The potency of the tested substance is obtained by comparing the effects of different doses of cod liver oil with those of the different doses of Standard, as was demonstrated in the example on p. 38.

B. Occurrence of xerophthalmia as a criterion for the determination of vitamin A.

α. Curative Method

The condition which sometimes develops in the eyes of rats which are fed long enough on a vitamin A deficient diet to exhaust their reserves of that factor has been variously named ophthalmia, xerophthalmia and keratomalacia. The eyelids first become bare and swollen. Then they bleed and form an exudate which may make it impossible for the rat to open its eyes. The eyeball itself becomes affected, a mass of pus may form in it and the sight of the eye may be completely lost; if it is, it cannot be recovered. If, however, vitamin A is given in the early stages of the condition recovery is complete.

The difficulty of using the onset of ophthalmia as a sign that the rat's vitamin A reserves are exhausted and that it is therefore ready to be used in an experiment for the determination of vitamin A in any substance, is to decide on the degree of severity of this condition which it is useful to take as the criterion. Certainly it should be some stage before the eye itself is attacked; a good stage to take is that where bleeding has just begun. It probably matters very little which stage is taken, provided that the worker or workers on any one experiment can recognise and use the same stage.

(*a*) *The preparatory period.*—Rats such as those used in the "increase in weight" method are suitable for this also. Similar laboratory conditions of housing and diet may be used. The

weighing of the rats is also advisable, even though it is not to be used as a criterion of the experiment.

(*b*) *The test period.*—The same methods of dosing may be used as in the "increase in weight" method. The measurement to be made is the time taken to cure the ophthalmia developed in the preparatory period. Here another difficulty presents itself, the difficulty of deciding on which day each rat may be considered to be cured, but again some agreement can be made between the workers on any one experiment so that the error of diagnosis of cure may be as small as possible.

It should be possible to construct curves of response relating the time taken to effect a cure and the dose of vitamin A given. The writer is not aware that anyone has so far constructed any.

There should be four or five groups of animals, two of which are given graded doses of the International Standard and the others graded doses of the substance to be examined. There seems to be a general opinion that four or five times as much vitamin A is required to cure ophthalmia as is required to bring about an increase in weight in rats which have ceased to grow on a diet deficient in vitamin A.

(*c*) *Working out the result.*—The number of days taken to effect a cure by the rats of each group are averaged and the averages compared as in the example of the "increase in weight" method on pp. 38-44. *b* in this experiment is always negative.

β. Preventive Method

The experiment to be carried out by this method is arranged exactly like the "prophylactic" "increase in weight" method, the measurement taken being the number of days which elapse before the development of the desired stage of ophthalmia. The result is worked out in a similar way.

C. Changes in the vaginal contents as a criterion for the determination of vitamin A.

The normal œstrous cycle of a rat extends over a period of 5–6 days. It may be described briefly as a period of about 3 days (diœstrus) when the vaginal contents consist of millions of leucocytes and a few nucleated cells with a little mucus, followed by a period of about 6 hours when there are no leucocytes but thousands of nucleated cells, then a period of about 24 hours when the nucleated cells are mixed with about equal numbers

of keratinised cells ("cornified" cells), and then a period of 24 hours when the whole of the contents of the vagina appear to consist of these keratinised cells. Leucocytes then reappear, at first mixed with the keratinised cells, and later alone (Fig. 5). It is normally a consequence of ovulation and does not occur in ovariectomised rats (Long and Evans, 1922).

When rats are fed on a diet deficient in vitamin A and their reserves of that factor are becoming exhausted, keratinised cells are found in the vagina day after day whether they have been ovariectomised or not. If they are then given vitamin A the keratinised cells in time disappear, and in uncastrated animals normal cycles reappear (Evans and Bishop, 1922, and many later investigators, amongst whom may be mentioned Hohlweg and Dohrn (1930) who suggested the term "Kolpokeratose" for this condition).

The difficulty of applying this method to the determination of vitamin A is that, although the response of the rat to large doses of that factor is well marked, it is difficult to estimate small responses to small doses. Some definite criterion has to be decided upon so that measurements may be made on individual animals and the measurements for the group averaged. An early attempt by the writer to use this for the determination of vitamin A by means of young rats failed, for many of them became steady in weight before the vagina opened. With older rats, however, the method was more successful.

It has been suggested by Baumann and Steenbock (1932) that this criterion might be used for the determination of vitamin A by the use of mature rats and that the same rats might possibly be used over and over again.

Moll, Dalmer, Dobeneck, Domagh and Laquer (1933) used this symptom of vitamin A deficiency as the basis of a method for determining the factor in cod liver oil and foodstuffs. By an ingenious graphical representation of the course of the cure and its duration, they measured the effect of a single dose on each animal and averaged results from animals given the same dose. They decided that each rat's behaviour in its first use for the test was more erratic than its behaviour to subsequent doses and the first result with any one rat should therefore be discarded. Even when this precaution was observed, they decided that the accuracy of the test was not greater than that obtained by the "increase in weight" method, but that, being specific for vitamin

A, it made a good biological control of the "increase in weight" method.

The following method was worked out by Coward, Cambden and Lee (1935):

(*a*) *Preparatory period.*—Rats of about 140g. weight or more which have grown up on a diet only moderately rich in vitamin A are used. Smears of the vaginal contents are examined daily, a small spatula being inserted in the vagina, pressed against the wall and withdrawn with some of the contents of the vagina adhering. This material is rubbed off on to a small drop of water on a microscope slide, at once examined under the low power (two-thirds) of the microscope and a diagnosis made. No staining is necessary. Thus if the first smear proves unsatisfactory a second can be made immediately. When it is established that the rats are having cycles regularly and are therefore probably healthy animals, they are given a vitamin A-deficient diet until keratinised epithelial cells are found in the vagina for 10 successive days. They are then considered ready for the test.

(*b*) *The construction of a curve of response relating the number of days elapsing before the disappearance of keratinised cells from the vagina, and dose of vitamin A given.*—As the rats become ready for test, the first 7 are given in turn one dose only of 5, 10, 20, 40, 60, 100 and 200mg. respectively of a certain sample of cod liver oil. The next 7 rats are given similar doses in turn also, and the next until 10 rats have received 5mg., 10 have received 10mg. and so on. Vaginal smears are examined daily, Sundays included, and the number of days elapsing before the keratinised cells disappear from the vagina is taken as the measurement required for the test. The periods required by the different rats given any one dose are averaged and the averages from the different groups of rats are plotted against the doses of cod liver oil given. The lower doses may be insufficient to bring about a positive reaction in all the rats to which they were given. Coward found that if a rat did not respond in 10 days it never did, so for the purpose of averaging the results from a group of rats each rat which did not respond in 10 days was given the value 10. With this convention, a fairly smooth curve of response was obtained (Fig. 7). It was logarithmic in shape and was represented by the equation $y=13{\cdot}2-4{\cdot}5 \log. x$, where y=the number of days elapsing between the giving of the dose

and the disappearance of keratinised cells from the vagina, and x was the single dose (mg.) of cod liver oil given.

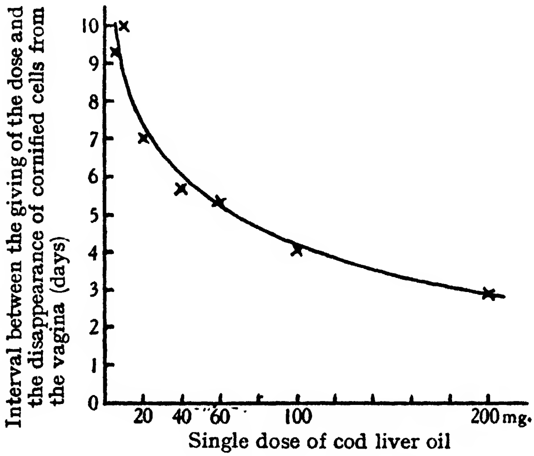

FIG. 7.—Curve of response relating the dose of vitamin A to the time taken to restore the normal condition of the vaginal contents.

Having determined that the shape of the curve of response is logarithmic then the test can be arranged as it is in the "increase in weight" method.

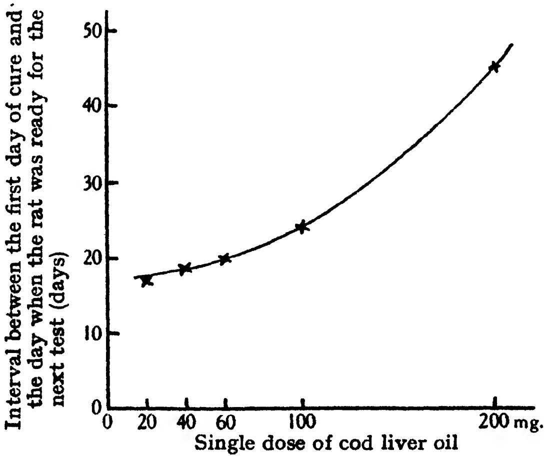

FIG. 8.—Curve of response relating the dose of vitamin A to the "duration of the cure" of the condition of the vaginal contents.

(c) *The duration of the "cure" from a single dose of vitamin A.*—Smears from the rats in the construction of Coward's curve of response to a single dose of cod liver oil were examined daily

until keratinised cells again appeared continuously in the vaginal contents, 10 days' continuous occurrence of these cells being the criterion adopted as evidence of vitamin A deficiency. A curve of response for the "duration of cure" was obtained, curvilinear in shape but not logarithmic (Fig. 8). It showed that the size of the dose of vitamin A given influences not only the rapidity of the response but also the duration of the rat's resistance to a subsequent shortage of vitamin A.

(*d*) *The possibility of using the same rats for several successive tests.*—Each rat in Coward's experiment which had responded to any given dose of cod liver oil was given no further dose of vitamin A until it had again reached the stage when it was considered ready for the test, *i.e.* when it had again shown keratinised cells in the vagina for 10 successive days. It was then given a dose of the same cod liver oil equal to the dose first given and the response to this second dose compared with the response to the first dose. It was found with all the doses that the rats took longer to respond to the second dose than to the first dose. They had become less sensitive to their doses of cod liver oil. Hence rats which are used a second time for this test must only be used for comparison with other rats which are being used a second time.

The accuracy obtainable by the method of determination was found to be less than that obtainable in the 3 weeks' "increase in weight" method.

Pugsley, Willis and Crandall (1944) have modified this method in certain respects; they have used younger animals (about 45 days of age), *i.e.* they were still growing, and ovariectomised them. They gave doses morning and night for two or more days and used as the criterion the period between giving the first dose, through the time of recovery (leucocytes in the vagina) up to the reappearance of keratinised cells. The curve of response became a straight line when the logarithm of the response was plotted against the logarithm of the dose given. Probably each of the modifications described helped to reduce the inaccuracy of the test which was less than that obtained by Coward. A further modification was shown to be beneficial in this method as well as in the "increase in weight" method, *i.e.* giving orally 0·5mg. of tocopherol per rat per day; larger amounts than this had no greater effect. Pugsley *et al.* did not determine whether their rats became more or less sensitive in successive tests; they

say that they can be used repeatedly for eight or ten assays so presumably the behaviour in the last assay was not obviously different from that in the first.

D. **Comparison between prophylactic and curative methods.**

As only one "end-point" is needed in the prophylactic method, it would appear that this method had a great advantage over the curative method which involves two end-points, one at the beginning of the curative period and the other at the end. It is also claimed as an advantage of this method that the rats do not have to bear the strain of the period when their reserves of vitamin A are nearly exhausted before they are given a dose of test substance or Standard for the curative part of the test.

Against the first argument in favour of the prophylactic method is the fact that individual rats vary so much in the amount of their vitamin A reserves that more rats would have to be used in a comparison of unknown and standard than would have to be used in a curative test where the rats are brought to a more or less similar state of depletion by the preliminary feeding on the vitamin A-free diet. That the rats are in a varied state of ill-health after their depletion period is evident from the fact that when given a small dose of vitamin A some rats continue to lose weight and die, whereas others recover and even grow slowly. If similar rats are given a larger dose of vitamin A, possibly one or two may die, but the majority will grow, though at different rates. The surprising point about this variation in response to doses of vitamin A is that, among the rats which respond and grow, it is no greater than the variation in growth of rats of similar age or weight in a normal colony. This was shown by Coward (1932) in an examination of over 2,000 experimental rats and nearly 500 normal rats from the same colony. Thus the only argument brought forward in favour of the prophylactic test seems to be based on a wrong assumption. The advantages of the curative method are numerous. It takes a shorter time and greater accuracy is obtainable by its use than by the prophylactic method.

E. **Comparison between (a) the "increase in weight" method, (b) the "xerophthalmia" method and (c) the "vaginal contents" method.**

The "increase in weight" method has the distinct advantage over the other methods in having a criterion that is easily

measured, though even this is not quite as accurate as one might at first imagine it to be, for a rat's weight fluctuates during 24 hours—it does not increase or decrease at a steady rate. This criterion is, however, much more accurately measured than either of the other criteria used for the determination of vitamin A, the error due to the uncertainty of whether xerophthalmia may be considered cured or not on any particular day being much greater than the error due to the fluctuation in weight of a rat during the day.

On the other hand, both the occurrence of xerophthalmia and the changes in the vaginal contents are thought to be due specifically to vitamin A deficiency and many people hold the opinion that a determination based on a reaction which is specific for the factor being measured is far better than a more accurate method based on a reaction such as increase in weight which is not specific for any one substance.

4. Physical Properties of Vitamin A by means of which it may be Measured

Vitamin A, as it occurs in liver oils and concentrates, shows an apparently characteristic absorption band in the ultra-violet region with a maximum at 328mμ.

The blue colour which is produced when a liver oil or concentrate is treated with arsenic or antimony trichloride is probably due to the formation of a derivative of vitamin A.

Either of these properties could be used for the measurement of vitamin A if it were certain (*a*) that no other substances acted in a similar manner and thus enhanced the measurement, and (*b*) that no other substances interfered with the reaction and diminished the measurement. A great deal of work has been done to try to find ways of eliminating these possibilities, but the difficulty is not yet wholly solved.

(A) *The absorption of rays of wave-length* 328*mμ*.—In the earlier work the measurement of absorption at 328mμ was made on the oil itself. Then it was shown that the presence of unsaturated fatty acids raised the value obtained. Even blowing air or oxygen through the oil could quadruple the value. It therefore became obvious that the measurement should be made on the unsaponifiable part of the oil and not on the oil itself. It also became evident that different results were obtained when

different solvents were used in the determination. A small subcommittee was appointed by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute to investigate the methods already in use for measuring the intensity of absorption at 328mμ by oils and their concentrates, and to make recommendations for the guidance of other workers in this subject. The members of the subcommittee examined six different samples of cod liver oil and one concentrate, measuring the intensity of absorption at 328mμ of the oil itself and also of the unsaponifiable part of each oil, prepared in different ways. The cod liver oils chosen included medicinal oils, oils intended for cattle or poultry feeding, one crude oil and one "coast cod" oil. The results obtained on these oils have been published in some detail in a Special Report, No. 202, of the Medical Research Council. The conclusions reached by the members of the subcommittee who examined these oils were summarised thus :

(i) Estimations on cod liver oils should be made on the unsaponifiable fraction of the oil.

(ii) Cyclohexane or ethyl alcohol and not chloroform should be used as solvent.

(iii) Only a method of saponification approved for the purpose should be used.

The method of saponification selected by the International Conference on Vitamin Standardisation, 1934, is the following :

One gram of oil is saponified with 10cc. N/2 freshly prepared alcoholic KOH, by boiling until clear (time needed about 5 minutes). 20cc. water are added, the whole transferred to a small separator and extracted with two quantities of 25cc. ether (peroxide-free). The ethereal extracts are washed first with water (10–20cc.), then with 10–20cc. N/2 KOH and again with water, while rotating gently without shaking. The ethereal solution is then shaken thoroughly with two quantities of 10cc. water, after which it is filtered into a flask, the ether evaporated almost to dryness and the residue dissolved in ethyl alcohol or cyclohexane and made up to the concentration required for the particular instrument in use. A preliminary test on the original oil will indicate the amount both of oil and of solvent which will be necessary.

Pure cyclohexane, suitable for spectrographic examination, should have the following properties: $d^{20°}_{4°}=0{\cdot}7784$; B.P.

81·4° C.; F.P. 6·5° C.; it should be almost completely transparent in the region of 328mμ and exhibit no trace of discontinuous absorption.

Conversion of values found spectroscopically into International units of vitamin A activity.—The intensity of absorption of an oil at 328mμ is expressed in the form $E^{1\%}_{1cm.}=x$. That is, the solution examined must be of a convenient strength to get an accurate reading, but the value must be calculated to a concentration of a 1% solution of the oil (or the corresponding amount of unsaponifiable matter) in alcohol or cyclohexane, and the solution must be examined in a cell of 1cm. thickness. E is the log. of the ratio between I_0, the intensity of the incident light and I, the intensity of the emergent light. Thus, suppose the value of $E^{1\%}_{1cm.}$ for a particular oil is given as 1. This means that only 10% of the incident light is transmitted by a depth of 1cm. of a 1% solution of that oil; for, given log. $I_0/I=1$, therefore (since log. $10=1$) the intensity of the incident light is ten times the intensity of the emergent light; or the emergent light is 10% of the incident light and a depth of 1cm. of a 1% solution of the oil has absorbed 90% of the incident light. Similarly, if the value of $E^{1\%}_{1cm.}$ were given as 1·3, then log. $I_0/I=1{\cdot}3$ and, since log. $20=1{\cdot}3$, the intensity of the incident light is twenty times the intensity of the emergent light; thus the emergent light is only 5% of the incident light, and a depth of 1cm. of a 1% solution of the oil has absorbed 95% of the incident light.

If a few more values are substituted for $E^{1\%}_{1cm.}$ and the corresponding percentages of light absorbed are determined and all the percentages so obtained are plotted against the corresponding values of $E^{1\%}_{1cm.}$, it is found that the relationship is curvilinear. This may appear disturbing until it is realised that the percentage of light absorbed is not proportional simply to the concentration of the absorbing substance. That the values for $E^{1\%}_{1cm.}$ are proportional to the concentration of vitamin A in a solution (provided no interfering substances are present) may be deduced from the general formula

$$E = \log. I_0/I = ecd, \text{ where } e \text{ is a constant}$$

for the substance under examination, c is the concentration and d is the thickness of the layer of solution examined. In the measurement of vitamin A, e is a constant, d is always 1cm. and therefore constant; therefore $E^{1\%}_{1cm.}=Kc$, that is $E^{1\%}_{1cm.} \propto c$ or $E^{1\%}_{1cm.}$

varies directly as *c*. Thus the measure, $E^{1\%}_{1cm.}$, may be taken as a measure of the vitamin A content of liver concentrates.

The Permanent Commission on Biological Standardisation (1934) adopted provisionally the factor 1,600 for converting the E value of an oil into the biological value in terms of the International unit. The evidence on which the decision to do this was based is summarised in the Special Report, No. 202, of the Medical Research Council. Since then, however, a serious dispute as to the general applicability of this factor has arisen, many American workers claiming that a factor of 2,000 was more generally correct. In order to test this, the vitamin A subcommittee of the Accessory Food Factors Committee in 1935 (Hume, 1937) and 1937 (Hume, 1939) organised a collaborative experiment in which workers in nine different laboratories determined the potency of a sample of halibut oil, a concentrate made from it and the U.S.P. reference oil. The Standard of reference was the International Standard consisting of a solution of pure β-carotene in oil, of such a strength that 1 gram contained 500 International units or 300 micrograms (300μg.) of β-carotene. The spectrophotometric determinations were made by Professor R. A. Morton and the conversion factor worked out to be 1,570 for the halibut oil and 1,820 for the U.S.P. oil. The concentrate proved to be unstable so the result from it was not considered. It was therefore recommended that the provisional value of 1,600 should be retained until further information was available. In 1939 (Hume, 1943) a further experiment was made in which most of the previous workers again collaborated. A sample of vitamin β-naphthoate was determined in comparison with the International Standard of β-carotene, and spectrophotometric determinations were made at the end of each biological test as well as at the beginning. The vitamin A β-naphthoate had the value $E^{1\%}_{1cm.}$ 325mμ=0·094. The biological values ranged from 97 to 260 I.U. per gram with an average of 166 I.U. and limits of error (P=0·99) 89–112%. Thus the conversion factor according to this experiment is 166÷0·094=1,770, which lies between the two values obtained previously. The three values 1,570, 1,770 and 1,820 were found not to differ significantly. They were therefore pooled and the value 1,740 obtained with limits of error (P=0·99) of 93–107%. The value 2,000 has mostly been found in the United States by the use of the U.S.P. oil of reference which had been found in 1931, by exhaustive

tests against the 1931 International β-carotene standard, to contain 3,000 I.U. per gram. When, however, the vitamin A subcommittee examined this oil in comparison with the 1934 International Standard of β-carotene it was found to contain 2,619 instead of 3,000 I.U. per gram, a discrepancy closely comparable with that found between the conversion factors of 1,740 and 2,000. This discrepancy may have arisen through an imperfect comparison between the 1931 and 1934 Standards of reference, or it may be due, at least in part, to deterioration of the oil for which there is spectroscopic evidence. It is a matter more of practical than of academic interest. The U.S. Pharmacopœia adopted the International Unit of vitamin A activity as its unit in 1934 and stated the value of its reference cod liver oil as 3,000 U.S.P. units per gram. But it seems to have been clearly shown that the U.S.P. oil does not in fact contain 3,000 I.U. Perhaps, therefore, the simplest solution would be to recognise that the U.S.P. and the International units are not in fact equal and that the former has only about 2,619/3,000 or about seven-eighths of the value of the latter. Then the respective conversion factors can remain at 2,000 and 1,740.

(B) *The "Blue Value" of vitamin A*.—How far the "blue value" of a vitamin A preparation is a true measure of its vitamin A content depends on the nature of the preparation and the way in which the blue value is determined. The student is referred to the appropriate sources of information for details of method. The writer is indebted to Professor R. A. Morton for the following summary of the position. The intensity of the blue colour given by rich oils and concentrates runs very parallel with the intensity of absorption at 325mμ measured directly. The blue solution exhibits a well-defined absorption band with maximum near 617–620mμ and the absorption curve has a shoulder near 580–583mμ of intensity just over half that of the main maximum. The colour test intensity is best determined either (*a*) spectrophotometrically at 617–620mμ (*e.g.* by using a visual spectrophotometer) or (*b*) photoelectrically, using a filter transmitting selectively around 620mμ.

Less potent oils, *i.e.* those containing fewer than some 8,000 I.U. per gram, frequently give anomalous colour tests, *e.g.* the colour is more purple than with rich oils. The intensity of absorption at 580–583mμ is more than half that at 617–620mμ. At potency levels below 2,000 I.U. per gram, the tint is further-

more affected by a displacement of the selective absorption. Thus in cod liver oil, the maximum may be at 603mμ and the shoulder at 570–572mμ. These difficulties may, however, be avoided by carrying out the determinations on non-saponifiable fractions prepared with due care. The colour test may be useful for preparations of low potency in which the ultra-violet absorption is masked by irrelevant absorption. In these the Carr-Price reaction may be unusually transient or other substances present may give interfering colours. These difficulties can usually be overcome by *ad hoc* calibrations made by adding known amounts of vitamin A to similar preparations. Carotenoids which give a blue colour of about one-tenth the intensity of that given by vitamin A can be determined by direct spectroscopy, if necessary after chromatographic separation. Many oils from fresh-water fish and a number of important commercial oils (*e.g.* ling cod) exhibit a colour test maximum at 693mμ due to vitamin A_2. In "ling cod," "black cod" and "red cod" liver oils from the Pacific Coast of North America, the 693 mμ absorption may be one-seventh that at 617–620 mμ. The colour test is then indispensable for the recognition of such quantities of A_2 and for the correction of estimates based on the ultra-violet absorption. Many workers consider that in the present state of knowledge it is reasonable to add the A_2 estimate to the A estimate in assessing the medicinal value of an oil. Vitamin A_2 besides showing the 693mμ maximum may also exhibit a shoulder near 648mμ analogous to the 583mμ shoulder of A. The interpretation of this is uncertain.

These notes apply mainly to natural oils. Highly processed products and materials worked up or prepared for research purposes may give rise to further difficulties, *e.g.* kitol from whale liver oil, anhydro-vitamin A and A_2, isoanhydro-vitamin A and sub-vitamin A, retinene and retinene$_2$ (the aldehydes from A and A_2).

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CHAPTER IV

THE DETERMINATION OF VITAMIN B_1

VITAMIN B_1 or aneurin is the name adopted by the Accessory Food Factors Committee of the Lister Institute and Medical Research Council and by the Biochemical Society for the factor which is essentially the antineuritic factor. It prevents and cures beri-beri in man, polyneuritis in pigeons and rats and is essential for the growth of the rat. In the United States of America it is still called vitamin B by some workers, but more often it is called thiamin. It has been prepared in the crystalline state as hydrochloride and sulphate by several workers and synthesised by three different methods. The original adsorption product

from an aqueous extract of rice polishings proved very satisfactory as the International Standard of reference, but when the synthetic vitamin became available it replaced the adsorption product.

I. The International Standard of Reference and the Unit of Vitamin B_1 Activity

The Standard of reference for vitamin B_1 which was recommended in 1934 by the Permanent Commission for Biological Standardisation of the Health Organisation of the League of Nations was the same as the one adopted in 1931 by the same Commission as a provisional standard of reference. It was a particular sample of an adsorption product on fuller's earth of an extract of rice polishings, and the International unit of vitamin B_1 activity was the amount of activity contained in 0·01g. of this particular preparation.

It has now been replaced by a particular sample of crystalline vitamin B_1 hydrochloride prepared synthetically. It was compared for biological activity with the standard adsorption product by workers in eighteen different laboratories in Europe, the United States of America and Japan. The methods used were the rat-growth, rat-curative (convulsions), rat-bradycardia, pigeon curative, chicken prophylactic and catatorulin. Thiochrome and colorimetric (of Kinnersley and Peters) methods were also used. The rather higher results obtained by the rat-growth method than by the other methods led to some interesting speculations, but it was decided to take the average of all the values obtained, and finally it was agreed that pure synthetic vitamin B_1 be adopted as International vitamin B_1 Standard and that the International unit be defined as the vitamin B_1 activity of $3\mu g$. of the pure material.

The Standard preparation in use at the present time (1945) contains no water of crystallisation and is stable when protected from access of moisture. Its criteria of purity are:

Colourless monoclinic plates, m.p. 246–7° (decomp.)
Picrate: yellow needles, m.p. 208° (decomp.)
Picrolonate: dimorphous:

- (*a*) yellow needles, m.p. 165° (decomp.)
- (*b*) yellow prisms, m.p. 226° (decomp.)

Absorption spectrum determined by E. R. Holiday:

Extinction Coefficient of the Standard in Aqueous Alcohol.

(Density 0·9351 at 20° C. containing $\frac{N}{100}$ HCl.)

λ.	K.	λ.	K.	λ.	K.
2300 Å	23·65	2525 Å	39·56	2750 Å	20·69
2325	27·03	2550	38·57	2775	17·46
2350	28·15	2575	37·45	2800	13·52
2375	35·47	2600	36·04	2825	9·85
2400	39·41	2625	34·91	2850	6·62
2425	42·09	2650	33·22	2875	4·65
2450	43·36	2675	30·97	2900	3·52
2475	42·52	2700	27·87	2925	2·96
2500	40·83	2725	24·36		

The standard preparation has been distributed into small tubes of non-alkaline resistance glass, each containing approximately 20 milligrams. After being kept for 14 days over phosphorus pentoxide *in vacuo*, during which no loss of weight was recorded, the tubes were filled with pure, dry nitrogen gas and sealed by the flame. They are stored continuously at −2° C. in the dark.

Indications of the amounts necessary in certain tests, and directions (which should be followed exactly) regarding the use of the standard in biological determinations are included in the memorandum which accompanies the first batch of the Standard sent to any worker.

A. **The need for a simultaneous test of the Standard of reference whenever a determination of vitamin B_1 is made.**

There are no records in the literature of one particular substance having been examined repeatedly to see how far the results of successive determinations of its vitamin B_1 content might vary, even when the conditions of the tests were thought to be the same. During a period of five years, however, the writer used the International Standard of reference for determining the vitamin B_1 potency of different substances, which has always involved a test on the Standard simultaneously with each determination made. Twenty-one tests on the original Standard were thus made, the same dose 0·03g. (3 units) being used in all the tests. The results may be seen in Table II.

TABLE II

THE VARIATION IN THE PERCENTAGE NUMBER OF PIGEONS IN DIFFERENT GROUPS CURED OF RETRACTED NECK AND THE AVERAGE DURATION OF THE CURE EFFECTED BY A SINGLE DOSE, 0·03G. (THREE UNITS) OF THE ORIGINAL INTERNATIONAL STANDARD ADSORPTION PRODUCT

	Date of test.	No. of pigeons given 0·03g. of the Standard.	No. of pigeons cured.	Percentage of pigeons cured.	Average duration of cure (days).
1	October, 1932	23	11	47·8	3·7
2	October–November, 1932	15	7	46·7	2·1
3	January–February, 1934	16	8	50·0	3·5
4	June–September, 1934	11	6	54·5	4·5
5	October–December, 1934	14	9	64·3	5·3
6	February, 1935	9	3	33·3	3·3
7	April–May, 1935	9	6	66·7	3·2
8	July, 1935	8	6	75·0	4·7
9	September, 1935	8	4	50·0	4·2
10	January–February, 1936	9	6	66·7	3·3
11	April, 1936	9	6	66·7	4·3
12	May, 1936	7	4	57·1	2·8
13	May, 1936	9	4	44·4	5·0
14	July, 1936	9	5	55·6	7·2
15	August, 1936	12	8	66·7	6·2
16	September, 1936	7	6	85·7	7·3
17	October, 1936	9	7	77·8	6·4
18	November, 1936	10	7	70·0	4·7
19	December, 1936	7	3	42·9	6·0
20	January, 1937	5	3	60·0	10·7
21	March, 1937	10	6	60·0	4·2

As the percentage of birds cured by the same dose, 3 units of vitamin B_1, varies, it is evident that a simultaneous test of the Standard must be made whenever the vitamin B_1 potency of a substance is to be determined.

Fluctuations in the average duration of the cure of retracted neck in pigeons are also found (Table II).

As the average duration of the cure of retracted neck in different groups of pigeons (each pigeon having been given 3 units of vitamin B_1) varies, it is evident that a simultaneous test of the Standard must be made whenever the vitamin B_1 potency of a substance is to be determined.

It has been suggested by Lassen (1936) that the average "duration of cure" of the birds should include the value 0 for

those birds which were not cured, but this cannot be considered a sound procedure since there can be no value in this record of response less than 0 and any value recorded as 0 might have really been less than 0, if it had been possible so to record it. Thus the only way to use the criterion "duration of cure" is to use it as its name implies, the duration of the cure effected by doses of vitamin B_1.

Fluctuations in the average increase in weight of groups of rats in response to doses of the International Standard are also found. Table III gives results obtained during the last five years in the writer's laboratory. The student is recommended to plot these results on a sheet of graph paper, males, females and simple and weighted averages. Since it has been shown that male and female rats respond equally to vitamin B_1, it is permissible to weight the average responses of male and female rats according to the number of each in the group.

The average increase in weight in 3 weeks from 4 or more rats given 3μg. (1 unit) of the International Standard varied from $-5{\cdot}75$g. to $+6{\cdot}95$g., and from 4 or more rats given 6μg. (2 units) of the International Standard it varied from 4·50g. to 17·50g. The groups of animals (4 or more) are certainly small, but Coward, Burn, Ling and Morgan (1933) have shown that a smaller number of rats is required for a given degree of accuracy in a vitamin B_1 determination than in a vitamin A determination. It is obvious from these figures that a simultaneous test of the Standard of reference must always be made whenever the vitamin B_1 potency of a substance is to be determined.

B. The general arrangement of the test for a determination of the vitamin B_1 potency of a substance in terms of the International Standard.

The same principles must guide the general arrangement of the test for a vitamin B_1 determination as for a vitamin A determination. It must be the object of the experimenter to find a dose or doses of the substance examined which gives a response equal to that given by a dose or doses of the Standard examined at the same time on similar animals. The responses to be aimed at are submaximal and preferably about half-way between no response and "complete" response. Thus whether pigeons or rats are the test animals, if no information of the probable

TABLE III

THE VARIATION IN THE RESPONSE OF RATS TO DAILY DOSES OF THE INTERNATIONAL STANDARD OF VITAMIN B_1

Date of test.	Daily dose, 1 unit (3µg.) of the current International Standard.					Daily dose, 2 units (6µg.) of the current International Standard.				
	No. of ♂'s.	Average increase in weight in 3 weeks. g.	No. of ♀'s.	Average increase in weight in 3 weeks. g.	Average increase calculated as if from equal numbers of ♂'s and ♀'s. g.	No. of ♂'s.	Average increase in weight in 3 weeks. g.	No. of ♀'s.	Average increase in weight in 3 weeks. g.	Average increase calculated as if from equal numbers of ♂'s and ♀'s. g.
June, 1939	3	0·3	2	−11·0	−5·35	3	7·0	2	2·0	4·50
July, 1939	3	−4·0	2	−4·5	−4·25	3	9·0	2	9·0	9·00
December, 1939	3	1·0	3	−0·3	0·33	4	9·3	4	13·5	11·40
December, 1939	5	−2·6	2	−8·5	−5·55	5	7·2	3	6·7	6·95
May, 1941	5	6·6	3	7·3	6·95	5	20·8	3	11·0	15·90
July, 1941	4	2·0	4	2·5	2·25	4	11·5	4	9·5	10·50
August, 1941	5	6·6	3	0·3	3·45	5	11·4	3	11·3	11·35
November, 1941	5	−1·4	3	−2·3	−1·85	5	6·8	3	6·7	6·75
December, 1941	4	−5·5	3	2·0	−1·75	5	6·6	3	5·2	5·90
January, 1942	2	8·5	3	1·7	5·10	2	21·5	3	11·7	16·60
February, 1942	5	6·4	3	1·7	4·05	5	15·2	3	11·0	13·10
March, 1942	5	1·0	3	2·0	1·50	5	8·6	3	11·7	10·15
June, 1942	4	−5·0	4	−1·3	−3·15	3	2·3	4	7·5	4·90
July, 1942	2	−6·5	3	−5·0	−5·75	2	11·0	2	8·0	9·50
August, 1942	7	−0·4	2	−4·5	−2·45	5	10·2	3	10·3	10·25
September, 1942	5	1·8	4	0	0·90	6	14·5	4	13·0	13·75
January, 1943	5	−5·6	2	−3·0	−4·30	6	5·2	1	7·0	6·10
January, 1943	5	1·8	2	−5·5	−1·85	5	7·4	2	2·5	4·95
June, 1943	9	−2·1	6	−0·5	−1·30	8	7·9	6	6·7	7·30
October, 1943	4	−0·8	3	−1·0	−0·90	4	9·5	4	6·8	8·15
October, 1943	—	—	—	—	—	4	12·5	1	4·0	8·25
November, 1943	5	4·2	4	5·3	4·75	4	14·0	4	16·5	15·25
March, 1944	4	2·5	2	1·5	2·00	5	14·6	2	12·0	13·30
March, 1944	3	6·7	4	4·5	5·60	4	17·5	4	17·5	17·50

potency of the test substance is available, the animals as they become ready for the test should be distributed in turn into, say, five different groups, two of the groups being given doses of the International Standard in the ratio 2 : 1 and the other three groups being given doses of the substance to be examined in a very wide range of doses, say, 9 : 3 : 1. This preliminary test may be made on 4 or 5 pigeons in each group of a pigeon test, or 3 or 4 rats in each group of a rat test, but it should always be followed by a further test on four groups of animals, two of these being given doses of the Standard in the ratio of 2 : 1 and two others being given doses which, judging from the preliminary

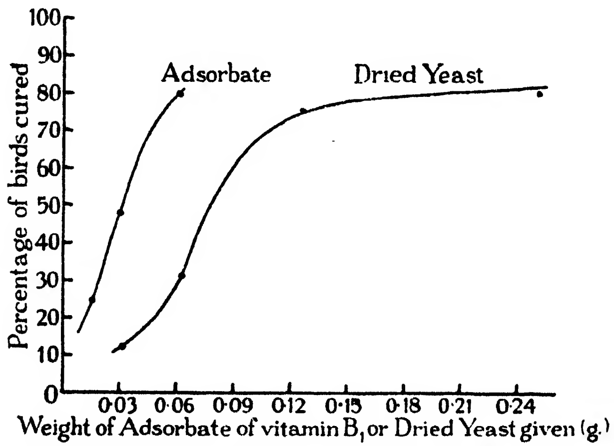

FIG. 9*a*.—Two examples of sigmoid curves, relating percentage of birds cured to dose of vitamin B_1 given.

test, will give results somewhere about those given by the two doses of the Standard.

The determination of the shape of the curve of response.—The method based on the cure of retracted neck of pigeons when given vitamin B_1 has been shown to be so inaccurate that the test is seldom used nowadays. It is an interesting method, however, since the curve of response is sigmoid in shape. Examples of such curves are shown in Fig. 9*a*. It is obviously desirable to get results as near as possible to 50% of cures. When the normal equivalent deviation corresponding to the proportion of birds cured (obtained from published tables, *e.g.* Pearson's *Tables for Statisticians and Biometricians*, Table I) is plotted against

the log. of the dose of vitamin given, the curve obtained is much more nearly a straight line and the best straight line can be calculated as described on p. 7 (Fig. 9*b*).

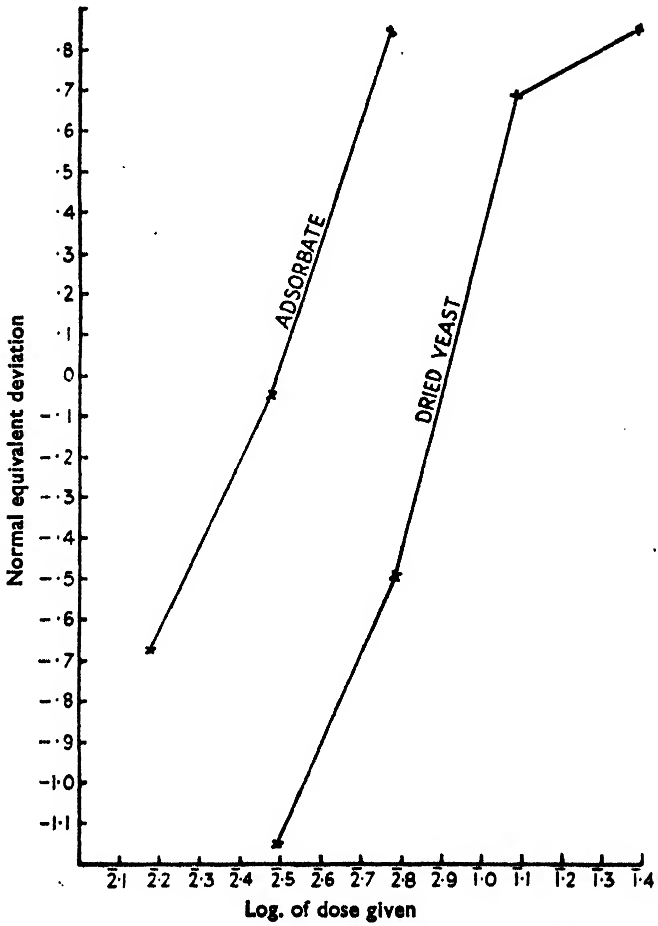

FIG. 9*b*.—The same results plotted as the normal equivalent deviation of proportion of birds cured (ordinates) against the logs. of the doses given (abscissæ).

The duration of the cure of retracted neck in the pigeons which survived in the test on the adsorbate (*i.e.* the period elapsing before retracted neck again occurred) gave a curve of response approximately logarithmic in shape (Fig. 10).

Similarly, in experiments on rats which have been given a vitamin B_1-free diet until they have become steady in weight

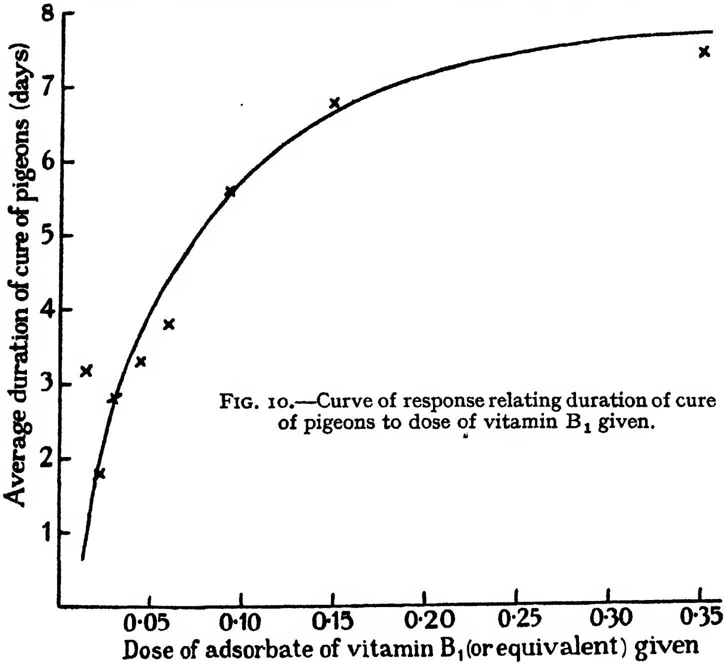

FIG. 10.—Curve of response relating duration of cure of pigeons to dose of vitamin B_1 given.

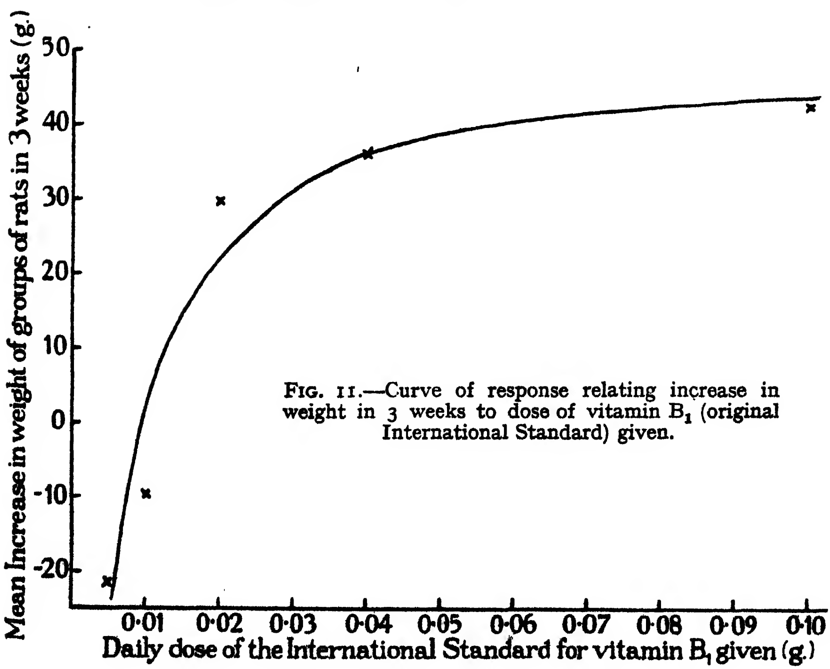

FIG. 11.—Curve of response relating increase in weight in 3 weeks to dose of vitamin B_1 (original International Standard) given.

and have then been given a daily supplement, a curve of response may be constructed relating increase in weight in a given time and dose of vitamin B_1 given. Useful doses for the construction of a curve of response are 0·005, 0·01, 0·02 and 0·04g. of the International Standard. This curve of response will probably be logarithmic. Separate curves of response for male and female rats may be constructed if desired, 4 or 5 animals being used in each group, but Coward, Burn, Ling and Morgan (1933) and Coward (1936) have shown that the curve of response for male rats is only slightly steeper than that for female rats. It is not significantly so and it is more convenient to average increases in weight from male and female rats than to evaluate the results separately (Fig. 11).

When convulsions in rats and bradycardia in rats are used as the criteria of vitamin B_1 deficiency, the shapes of the curves of response must be determined.

2. The Preparation of Pigeons for a Vitamin B_1 Determination

Some details of the carrying out of the "pigeon" test (cure of retracted neck) are retained in this edition since this was the first criterion used for demonstrating the presence of a vitamin in a preparation.

A. **Pigeons suitable for the test.**

It is not necessary to breed pigeons specially for vitamin work. The healthy stock of any reputable dealer is suitable.

The pigeons should be 300–350g. in weight for vitamin B_1 tests. Sex is apparently immaterial.

B. **Housing of the Pigeons.**

Cages about 3′ square by 6′ high are suitable for housing experimental pigeons. This size can accommodate about 10 birds conveniently. The cages may be arranged in series, side by side in the open air. The group of cages should be sheltered from the wind and rain. Moderate cold does not harm the birds. Each cage should have a doorway large enough for a worker to enter the cage. It is useful to have a passage closed by wire netting in front of the whole series of cages, so that if a bird escapes from its cage it may be recaptured in the passage.

As much as possible of the structure should be of wire mesh, about 0·5″ diameter. The floor of each cage should be of wire mesh, raised about 2″ from a tray which can easily be drawn out for cleaning. This gives the birds as little access to their fæces as possible. Birds and rats fed on a vitamin B_1 deficient diet will eat their fæces in order, presumably, to obtain the vitamin which is apparently generated by bacteria but not absorbed in the lower part of the gut.

The food and water of the pigeons should be placed in troughs just outside the cages, to which access may be obtained by a narrow slit in the wall of the cage.

Even with a floor made of wire mesh some part of the pigeons' fæces will be caught, and will be obtainable by the pigeons. The walls of wire mesh will also catch droppings from the birds clinging to them. Thus the more frequently the cages are cleaned the less chance will the birds have of obtaining vitamin B_1 from their fæces and the sooner will they develop polyneuritis.

C. **Diet of the pigeons.**

Polished rice (rice with the husks and embryo removed) is still the most generally used diet for producing polyneuritis in pigeons. Some workers wash it in running water and then dry it before using it—others consider this unnecessary. The pigeons eat plenty of this at the beginning of the experiment and supplies should always be greater than their need. A plentiful supply of clean water should be given fresh every day.

3. The Preparation of Rats for a Vitamin B_1 Determination

A. **Rats suitable for the test.**

Young rats weighing 50–60g. are suitable for determining vitamin B_1 by means of its influence on the weight of the rat. Male and female rats may be used. Coward, Burn, Ling and Morgan (1933) and Coward (1936) found that the response of male rats to doses of vitamin B_1 was not much greater than that of female rats. The difference, however, has since been found to be insignificant (unpublished results of the writer).

B. **Housing of the rats.**

Several rats may be kept together in one cage during the preparatory period, but during the test period each rat should be

kept in a separate cage. They may be housed in the ordinary wire cages of the laboratory but with one important addition. Grids of wire mesh of ½" diameter standing 1" high should cover the floor of each cage. This prevents the rats from having access to their fæces. Rats on a vitamin B_1 deficient diet are specially prone to eat their fæces which may contain vitamin B_1 and thus vitiate the test. Sawdust may be used in the bottom of the cage, or sheets of blotting-paper. The grids should be examined daily and changed if found to be soiled with excreta. The same warm temperature is required for rats in vitamin B_1 tests as for those in other vitamin tests.

C. **Vitamin B_1-free diet.**

The diet of rats for tests of vitamin B_1 must contain all the substances known to be necessary for growth except vitamin B_1. A suitable diet has the following composition :

Caseinogen	15 parts
Dextrinised rice starch..	79 ,,
*Agar-agar	2 ,,
Salt mixture (Steenbock's 40)..	4 ,,
Dried brewers' yeast autoclaved at 15 lb. pressure for 6 hours	25 ,,

In addition each rat should be given 5 or 6 drops of a good sample of cod liver oil twice a week. This should be dropped directly into his mouth, so that it is certain that each rat has received his required portion.

The caseinogen named "light white" of the British Drug Houses has been found suitable for tests on vitamin B_1. It is not impossible that it contains small amounts of this factor, but that it does not contain appreciable amounts of it has been shown by Coward, Key and Morgan (1929). It is probably a sodium caseinate. It makes a colloidal solution in water by shaking or beating with an egg-whisk. The "Physiological Caseinate," made by the Glaxo Laboratories Ltd., has also been found suitable for these tests.

Dextrinised rice starch, prepared as described in the vitamin A tests, is used for the same reasons for which it is used in those tests.

Agar-agar (powdered) used to be included in the diet to prevent constipation which might otherwise result from a shortage of vitamin B_1, but since the supplies available in this country

* Apparently not essential (see below).

during the recent war were required for more urgent purposes and it was learnt that the workers at the Lister Institute never used it, it has been omitted from this diet without apparently any harmful results.

Fresh water must be supplied each day, preferably in an inverted bottle with a narrow neck. Any water which is fit for human consumption may be used for the rats. Distilled water is not necessary.

Autoclaved yeast must be supplied plentifully in the diet. It cannot be assumed that the amount of dried yeast which has been found to be in abundance in a vitamin A-free diet will, when autoclaved, supply abundance of vitamin B_2, etc., in a vitamin B_1-free diet ; *e.g.* Coward, Burn, Ling and Morgan (1933) found that 8% of autoclaved yeast in a vitamin B_1-free diet did not supply enough vitamin B_2 to obtain the full effect of doses of the International Standard for vitamin B_1, although they obtained graded responses to graded doses of the Standard. When, however, 20% of the autoclaved yeast was used, ample amounts of vitamin B_2 were supplied and a steeper curve of response was obtained. This dried yeast was autoclaved in layers about 0·1″ thick at 120° C. for 6 hours. A direct comparison between dried yeast autoclaved thus and an untreated portion of the same sample showed that this process of autoclaving had destroyed about 50% of some factor other than vitamin B_1 in the dried yeast. Therefore, since 8% of dried yeast provides abundance of all the B vitamins, 20% of yeast, autoclaved as described, should provide abundance of the B vitamins other than B_1 with a fair margin of safety.

4. Criteria for the Measurement of the Response of Animals to Doses of Vitamin B_1

The cure of "retracted neck" in pigeons has been widely used in testing substances for the presence of vitamin B_1, but the degree of accuracy obtainable is low. When these birds are fed on a diet of polished rice and water, and kept in cages with screens of wire mesh raised about 2″ from the floor to prevent access to fæces, 40–50% will develop "retracted neck" in 3 or 4 weeks. The head suddenly jerks backwards so that the top of the skull rests in the feathers of the bird's back. It may recover its normal position again for a time, but when the condition

becomes worse the neck remains retracted. The head can be moved about a little from side to side and can be held in its natural position by an operator without apparently giving the bird any pain. If no dose of vitamin B_1 is given the bird will die within about 48 hours. If, however, a dose of, say, 0·5g. dried yeast is given in a little water by pipette into the bird's throat it recovers in about 12 hours. If after that it is given no more vitamin B_1, it will again develop a retracted neck in a few days, or it may suddenly die.

The birds ought to be looked at three times a day. When one is found with a retracted neck it may be given the desired dose, and put in a smaller cage alone and still in the open air. It should be given food (the vitamin B_1 deficient diet) and water. If it is cured within 24 hours that may be considered a 1-day cure. If it remains cured for another 24 hours, that may be considered a 2-days cure, and so on. Parts of days must be ignored unless the birds are watched all night both for development of retracted neck and for cure. The cure is quick as a rule but not sudden. The neck may regain its normal position for a short time and then revert to the abnormal position, but if the dose is really curing the bird the head will be normal in 24 hours. It really is useless to try to refine the calculation more than this.

Kinnersley, Peters and Reader (1928) found it useful to give a pigeon a small dose of glucose in water and then wait a few hours to see whether that would bring about a "spontaneous cure" of the bird before giving it a dose of test substance or of the Standard. A few birds may respond to this treatment and develop the true polyneuritis a few days later.

When rats are fed on a diet which contains all substances known to be necessary for growth except vitamin B_1 they will cease to put on weight in 1–2 weeks and if still no vitamin B_1 is given to them they will lose weight and die in 2–3 weeks. If, on the other hand, they are given a daily dose of a substance containing vitamin B_1 they will increase in weight at a rate proportional to the log. of the dose of vitamin B_1 given.

M. I. Smith (1930) has produced polyneuritis in rats by feeding them on a diet "so constituted as to include the thermostable factor but lacking in the antineuritic vitamin." He says this condition results almost invariably after 6–8 weeks of feeding on the deficient diet. Furthermore, the administration of a yeast concentrate containing the antineuritic vitamin brings about

prompt and complete recovery from the paralytic symptoms, the duration of the remission being from 3–15 or more days according to the size of the dose administered. The paralysis will recur again if no further vitamin B_1 is given but may again be alleviated. The paralytic condition is characterised by lameness of the hind- and fore-limbs, inco-ordination, spastic gait, cart-wheel and rolling movements. Intravenous injection of concentrates into a tail vein is recommended as being more certain than oral administration.

Drury, Harris and Maudsley (1930) and Birch and Harris (1934) have used the cure of bradycardia for measuring the vitamin B_1 content of a substance. When young rats are given a vitamin B_1 deficient diet their heart-rate falls. In 3 weeks it has generally fallen from about 500 or 550 per minute to 350 per minute. When a single dose of vitamin B_1 is given the heart-rate is increased, reaching the maximum obtainable on the given dose in about 24 hours, after which, if no further vitamin B_1 is given, the rate again declines. The rate is determined by means of an electrocardiograph.

A. **Cure of retracted neck in pigeons.**

This is the simplest biological test known for any vitamin. The preparation of the birds is simple, one dose only is administered to each bird and the result is available the next morning if a cure is the only figure required, or in a few days if the duration of cure is required.

(*a*) *Preparatory period.*—Pigeons suitably housed and given a diet of polished rice and water will usually develop retracted neck in 20–30 days if they do so at all. About 50% of pigeons never develop retracted neck and are therefore wasted in a vitamin B_1 test.

(*b*) *Dosing of the pigeons.*—The Standard and most test substances can be given to the pigeons very easily. The Standard in suitable dilution is drawn up into a pipette to which is attached about 2″ of fine rubber tubing. The free end of the tubing is inserted into the throat of the pigeon whose neck is held extended with the left hand, and the desired dose is allowed to run out.

Bulky substances of low vitamin B_1 content may be given by forcible feeding down a glass tube passed into the œsophagus of the bird. The food is finely ground and rammed down the tube by a narrow glass rod.

Occasionally it is found that a pigeon cannot tolerate a substance and will vomit it. This pigeon should be rejected. If all the pigeons behave in the same way to any particular substance, the pigeon test will have to be abandoned and a rat test tried.

(*c*) *Working out the result.*—(i) Suppose the following figures had been obtained in a determination of the vitamin B_1 content of a substance by the pigeon (cure of retracted neck) method.

Dose.	Percentage of birds cured.	Average duration of cure (days).
0·02g. Inter. St. (original)	30	2·1
0·04g. ,, ,,	60	3·1
0·1g. substance X	12	0·5
0·2g. ,, ,, ...	25	1·3
0·4g. ,, ,, ...	30	2·0

It is tempting to say from both methods of measuring the response that 0·4g. substance X was equal in vitamin B_1 potency to 0·02g. International Standard, but this would take no account of the information supplied by the three other groups of pigeons. Then calculate the potency as below.

Curve for Standard.

Percentage birds cured.	Normal equivalent deviation, y.	Dose of original Inter. St.	Log. dose, x.
30	−0·5244	0·02g.	$\bar{2}$·3010
60	+0·2533	0·04g.	$\bar{2}$·6021
	Diff. = 0·7777		Diff. =0·3011

The slope of the curve is $\frac{0{\cdot}7777}{0{\cdot}3011}=2{\cdot}583$.

The "mid-point" of the curve (or point with average values of x and y) is $\bar{2}$·4515, −0·1355.

The equation of the curve is

$$\frac{y-y_1}{x-x_1}=2{\cdot}583$$

i.e.
$$\frac{y-(-0{\cdot}5244)}{x-\bar{2}{\cdot}3010}=2{\cdot}583$$

therefore
$$y=2{\cdot}583x+3{\cdot}864x$$

Check this equation by substituting simple values for x:

e.g. when $x=\bar{2}{\cdot}3$ $y=-0{\cdot}5270$

,, $x=\bar{2}{\cdot}6$ $y=+0{\cdot}2479$

Curve for Substance X.

Percentage birds cured.	Normal equivalent deviation, y.	Dose.	Log. dose, x.	$x-\bar{x}$.	$y(x-\bar{x})$.	$(x-\bar{x})^2$.
12	$-1\cdot1750$	0·1g. X	$\bar{1}\cdot0$	$-0\cdot3010$	$+0\cdot3536750$	0·090601
25	$-0\cdot6745$	0·2g.	$\bar{1}\cdot3010$	0	0	0
30	$-0\cdot5244$	0·4g.	$\bar{1}\cdot6021$	$+0\cdot3010$	$-0\cdot1578444$	0·090601
	$-2\cdot3739$		$\bar{3}\cdot9031$		$+0\cdot1958306$	0·181202
	$\bar{y}=-0\cdot7913$		$\bar{x}=\bar{1}\cdot3010$			

$$b, \text{ the slope of the curve is } \frac{0\cdot1958306}{0\cdot181202}=1\cdot0873$$

The curve is

$$\begin{aligned} y&=1\cdot0873(x-\bar{x})+\bar{y} \\ &=1\cdot0873(x-\bar{1}\cdot3010)-0\cdot7913 \\ &=1\cdot0873x-0\cdot0313 \end{aligned}$$

Check this equation by substituting simple values for x:

e.g. when $x=\bar{1}\cdot0$ $y=-1\cdot1186$
" $x=\bar{1}\cdot6$ $y=-0\cdot4662$

and plot the curve and the points from which it was derived on graph paper.

Note that the slope of this curve is different from that for the Standard. Find out, sooner or later, by the method described in Chapter X whether the difference between the two slopes is significant.

The "mid-point" of the curve for the substance X (or point with average values of x and y) is $\bar{1}\cdot3010$, $-0\cdot7913$.

Curves with average slope must now be drawn through the "mid-points" of the two curves for Standard and substance X respectively, and the horizontal distance between them determined.

The average slope of the two curves is

$$\frac{2\cdot583+1\cdot087}{2}=1\cdot835$$

The curve with average slope, 1·835, through the "mid-point" of the curve for the Standard is

$$\frac{y-(-0\cdot1355)}{x-\bar{2}\cdot4515}=1\cdot835$$

therefore

$$y=1\cdot835x+2\cdot706$$

The curve with average slope, 1·835, through the "mid-point" of the curve for substance X is

$$\frac{y-(-0\cdot7913)}{x-\bar{1}\cdot3010}=1\cdot835$$

therefore $y=1\cdot835x+0\cdot4932$

The horizontal distance between these two curves (which is the log. of the ratio of the potencies of the two preparations) is

$$\frac{0\cdot4932-2\cdot7060}{1\cdot835}=-1\cdot2059$$
$$=\bar{2}\cdot7941$$
$$=\log.\ 0\cdot062244$$

The potency of substance X is therefore 0·062 of that of the Standard. That is, substance X contains 6·2 International units of vitamin B_1 per gram. This result is about 25% higher than that obtained by the rather casual comparison made at first. It is based on two and a half times the number of pigeons and therefore is probably nearer the true result.

Draw the curves with average slope on the graph of the original curves and check the result obtained above by measuring the distance between the parallel curves.

The figures for the duration of cure may be treated similarly, assuming that the duration of cure is proportional to the log. of the dose given. The student is advised to work this out for himself. The answer is 4·2 International units per gram, which is different from the 5 I.U. obtained by a casual inspection of the results and very different from the 6·2 I.U. obtained by calculation from the percentage of birds cured; but this was only an imaginary experiment, made up to show the importance of using *all* available information for the calculation of a result by using either of the two criteria. (See also Chapter XI.)

B. **Increase in weight of rats.**

(*a*) *Preparatory period.*—Rats require a shorter period in which to exhaust their reserves of vitamin B_1 than the period they require to exhaust their reserves of vitamin A or D. They generally become steady in weight in 10–14 days of feeding on the vitamin B_1-free diet. They should be weighed twice a week during this time and put on experiment within a day or two of the first fall in weight, for on a shortage of vitamin B_1 rats quickly lose weight and die.

(*b*) *The test period.*—There is no need to carry on a rat test for vitamin B_1 longer than 3 weeks. Indeed, Coward (1936) has

shown that a test carried on for 2 weeks gives a result almost as accurate as one carried on for 3 weeks. The accuracy obtainable in a vitamin B_1 test is certainly greater than that obtainable in a vitamin A test in the same length of time.

During the test period doses of the test substance and of Standard may be given daily or half-weekly. In three different experiments, one with dried yeast and two with different adsorbates of vitamin B_1 similar to the International Standard, Lindholm, Laursen and Morgan (1938) have found these two ways of giving the doses to produce equal responses.

Doses of test substances (if not too potent) may be weighed directly, transferred to a small dish and moistened to prevent scattering. Dried yeast, wheat embryo, etc., are conveniently given to the rats in this way.

Substances of lower potency may be mixed with the diet in the required percentage assuming that a rat of 50–60g. weight eats about 5g. food (dry weight) per day. Records of food consumed should then be made in order to determine the exact weight of test substance eaten during the full period of the test. Food-pots with turned-in rims should be used to prevent scattering. Ten grams of food may be given on the first day of the test, the part not eaten by the next morning weighed and made up to 10g. again or more if necessary. There should always be a surplus of about 2g. in the pot in the morning.

The rats may be weighed only once a week during the test period, but additional weighings on the day before and the day after the last weighing should be made and the three weighings averaged for the final weight. One additional dose should then be given.

(*c*) *Working out the result.*—(i) When two or three doses of the test substance have been given to different groups of rats, and two doses of the International Standard to two other groups, the average increases in weight of the rats of the different groups are calculated and compared. The following figures were obtained in a test carried out as described above :

Substance.	Dose, g.	No. of rats in test.	Mean increase in weight in 3 weeks, g.
X	0·5	5	15·4
X	1·0	6	29·0
International Standard	0·01	5	11·6
International Standard	0·02	6	23·2

It is evident that 0·5g. substance X contains a little more vitamin B_1 than 0·01g. of the International Standard; that is, 0·5g. X contains a little more than 1 International unit.

Similarly, 1·0g. substance X contains a little more vitamin B_1 than 0·02g. International Standard; that is, 1·0g. X contains a little more than 2 International units. But 0·5g. X is far from being equal to 0·02g. of the Standard. Thus the nearest approximation to the true vitamin B_1 potency of substance X that can be made from these figures is probably about 2·3 International units per gram.

(ii) The result may be calculated as follows:

Mean increase in weight of rats in 3 weeks.	Dose.	Log. dose.	No. of rats in group.
15·4g.	0·5g. X	$\bar{1}$·6990	5
29·0g.	1·0g. X	0	6
Diff.=13·6		Diff.=0·3010	
Slope of curve, b, $=\dfrac{13·6}{0·301}=45·18$			
11·6g.	0·01g. orig. Inter. St.	$\bar{2}$·0	5
23·2g.	0·02g. ,, ,, ,,	$\bar{2}$·3010	6
Diff.=11·6		Diff.=0·3010	
Slope of curve, b, $=\dfrac{11·6}{0·301}=38·54$			

The average slope of the curves is

$$\frac{45·18+38·54}{2}=41·86$$

The point with average values of x and y in the curve for substance X is $\bar{1}$·8632, 22·8. The point with average values of x and y in the curve for the Standard is $\bar{2}$·1642, 17·9. The curve with average slope, 41·86, through the "mid-point" of the curve for substance X is

$$\frac{y-22·8}{x-\bar{1}·8632}=41·86$$

therefore $$y=41·86x+27·53$$

The curve with average slope, 41·86, through the "mid-point" of the curve for the Standard is

$$\frac{y-17·9}{x-\bar{2}·1642}=41·86$$

therefore $$y=41·86x+94·75$$

The horizontal distance between these two curves is

$$\frac{27{\cdot}53-94{\cdot}75}{41{\cdot}86}=-1{\cdot}6058=\bar{2}{\cdot}3942=\log.\ 0{\cdot}024785$$

Therefore substance X has about 0·025 of the potency of the original International Standard, *i.e.* it contains about 2·5 International units per gram.

If the numbers of rats used for testing the Standard and substance X are different, the slopes of the two curves have to be weighted for the average thus:

No. of rats.	Dose.	Weight factors.
5	0·05g. X	$\frac{5\times6}{5+6}=\frac{30}{11}=2{\cdot}73$
6	0·1g. X	
6	0·01g. St.	$\frac{6\times7}{6+7}=\frac{42}{13}=3{\cdot}23$
7	0·02g. St.	

The average slope then is

$$\frac{45{\cdot}18\times2{\cdot}73+38{\cdot}54\times3{\cdot}23}{2{\cdot}73+3{\cdot}23}=41{\cdot}58$$

which in this experiment is not very different from the other average.

C. Cure of convulsions in rats.

The writer has had no success in attempts to produce convulsions in rats. Therefore the following details of this method are based on work by Birch and Harris (1934):

(*a*) *Preparatory period.*—If rats of 70–80g. weight are given no vitamin B_1 in their basal diet they will die without developing polyneuritic convulsions, but if given traces of vitamin B_1 (enough just to keep them alive) they will develop this symptom. Thus a very small dose of vitamin B_1 should be given to each rat during the preparatory period (Sherman and Sandells, 1931, Sebrell and Elvove, 1931). In 6–8 weeks the polyneuritic condition generally becomes evident (Birch and Harris, 1934).

(*b*) *Dosing the rats.*—Doses of test substance or of Standard may be given by mouth. If a comparison of concentrates is to be made, the doses may be injected intraperitoneally. As the rats become ready for the test they are distributed into, say, four

groups, two groups for doses of the Standard in the ratio 2 : 1 and two groups for doses of the test substance. The latter doses should be of a very wide range if no information is available of the probable potency of the substance, but when that information has been obtained, a narrower range of doses is tested against a fresh test of two doses of the Standard.

(*c*) *Working out the result.*—The average number of days that the rats remain cured after receiving a dose is calculated for each group. The most satisfactory determination is made when the average result from a dose of the Standard is equal to the average result from a dose of the substance under test. If no two average results correspond, then the calculation is made as in the increase in weight method, viz. by constructing curves from the pairs of results from the two doses of Standard and test substance respectively.

D. **Cure of bradycardia in rats.**

This method is based on the details given in the papers by Drury, Harris and Maudsley (1930), and Birch and Harris (1934).

(*a*) *Preparatory period.*—Young rats of about 40g. weight are given a vitamin B_1-free diet. After about 3 weeks electrocardiograms are taken. The unanæsthetised rat is stretched on a board, its head held firmly in a clamp, and its legs held out by slip nooses wound round cleats on the sides of the boards. One of the electrodes, a small needle, is placed under the skin of the right foreleg, the other at the lower end of the thorax. The needles are connected to a Matthew's portable electrocardiograph on which permanent photographic records are then taken. When the heart-rate is down to about 350 per minute the rat is ready for the test.

(*b*) *Dosing the rats.*—As the rats become ready for the test, two groups are given doses of the Standard in the ratio 2 : 1 and two are given doses of the test substance. If some information of the probable potency of the substance to be tested is available, then the doses may be in the ratio 2 : 1. If no such information is available, then a preliminary test must be made with doses in a wider range, say, of 3 : 1, followed by another test with doses in the ratio 2 : 1 more nearly equivalent to the doses of Standard, which must, of course, be tested again simultaneously with the two doses of test substance.

There is not enough material available for an estimation of the number of rats that should constitute each group in this test, but it is safe to recommend at least 5 rats for a group when two groups are used for the Standard and two groups for the test substance.

A single dose only of the Standard (or substance tested) is given to each rat. Its diet is the same as before. Its electrocardiogram is taken again 24 hours after dosing. It the rate has not increased a larger dose is tried. With a large enough dose, the rate rises to normal in 24 hours and then gradually slackens again. The increase in rate in 24 hours, and the time taken to slow down to the rate when the dose was given are both measures of the potency of the dose given. Each is roughly proportional to the dose of vitamin B_1 given. The relationship between dose and effect appears to be a straight line. Cures of 4 days' duration are a suitable basis of comparison at which to aim.

(c) *Working out the result.*—Since the relationship between effect and dose of vitamin B_1 given is a straight line, the potency of the substance tested may be worked out approximately by simple proportion sums from the average duration of the cures from the different doses of the Standard and of substance tested.

E. Influence on the vaginal contents of rats.

While vitamin B_1 was thought to consist of only one substance, Evans and Bishop (1922) and Macy *et al.* (1927) showed that in its absence, the cycle of changes in the contents of the vagina was upset, no keratinised cells being found there. Coward and Morgan (1941) showed that a deficiency of vitamin B_1 only produced this result and investigated the possibility of using this action as a criterion for the determination of vitamin B_1.

Rats of about 140g. weight which had never been mated were examined daily until found to have three normal cycles. They were then given the vitamin B_1-free diet of this laboratory and their vaginal contents examined daily until no keratinised cells had been found for 10 successive days. Four rats ceased at once to have cycles. The average number of days that elapsed after the vitamin B_1-free diet was first given and the 10 days' anœstrous period began was 14·25 with σ=5·43. Six or seven rats were kept in each cage (18″×12″×8″) on a grid of $\frac{1}{8}$″-mesh while given the basal diet only, but when on dose, each rat was kept in a separate cage.

As the rats became ready for the experiment they were distributed into 6 groups of 12 rats per group. The groups were given daily doses of 0·01, 0·02, 0·03, 0·04, 0·05 and 0·1g. per rat respectively of a vitamin B_1 adsorbate that contained 200 International units of vitamin B_1 per gram, *i.e.* the doses were 2, 4, 6, 8, 10 and 20 International units respectively. The powder was intimately mixed with finely ground dextrinised rice starch, partly to facilitate the daily measuring of doses and partly because the rats ate it more readily in this form. Each dose was given on a small dish mixed with a little water to prevent its being blown away. When a rat had shown 3 cycles, it was given no more vitamin B_1 until cycles ceased when it was again given a daily dose equal to the dose previously given. The whole procedure was repeated a third time, in order to see whether the sensitivity of the rats changed with successive treatments or whether they could be used repeatedly, regardless of the number of times they had already been used.

A curve of response was estimated for each of the three treatments. The first treatment gave the steepest curve, the third treatment the least steep curve. Moreover, the rats became increasingly sensitive to their respective doses; therefore in this method of assay, all the rats of a test would have to be first series, or second series or third series and not a mixture chosen indiscriminately. A sample of dried yeast assayed by this method was found to have a vitamin B_1 content of 50 International units per gram, which agreed fairly well with the result found by the "rat-growth" method which gave a result of 40 International units per gram.

The limits of error of this method, however, were estimated as 47–216% for P=0·99, which is less good than the limits of error 74–135% given in the British Pharmacopœia for the rat-growth method.

F. Comparison of the five methods.

The cure of retracted neck in pigeons, the cure of convulsions in rats, the cure of bradycardia in rats and the influence on the vaginal contents all appear to be specific for vitamin B_1 which gives these methods a great advantage over the "increase in weight" method with rats.

The retracted neck method requires 3–4 weeks to prepare the birds but only one day after the giving of each dose to get a

result, if the result is calculated from the percentage of birds cured. If the result is calculated from the duration of cure a few more days are necessary. If enough pigeons were kept in continuous preparation for the test the length of time required for a test would be decided by the longest period of cure of any bird. It is therefore a very quick test. It is also a very simple test, for the birds require very little attention during their preparatory period, beyond the giving of clean water and fresh rice each day and the cleaning of their cages twice a week. Also, only one dose is given to each bird.

The cure of convulsions in rats requires a longer time to prepare them for the test, but provided a large enough supply of rats is kept in preparation a test can be completed in as short a time as by the "cure of retracted neck in pigeons" method. The cure of bradycardia in rats requires about 3 weeks of preparation, but again, only one dose is given to each animal, unless the dose is seen next day to be ineffective, when a larger dose is given. The time taken to complete the test is about the same as that required by the "cure of convulsions in rats" method. The cost of the electrocardiograph would, however, be prohibitive for many laboratories. The influence on the vaginal contents is the longest and probably the least satisfactory of all the biological methods. The "increase in weight in rats" method requires about 2 weeks for the preparation of the rats, and then a daily or half-weekly dose of the substance under test or of the Standard, for 2 weeks at least. Hence a longer time is required for obtaining a result than is required by either of the other methods and more labour is required for the giving of doses. It is not specific for vitamin B_1 since so many other factors are required for an increase in weight. It is, however, very much more accurate than the pigeon method and probably also than the other two methods, but that consideration should not make one lose sight of the point that the other three criteria appear to be specific for vitamin B_1 while the "increase in weight" is not.

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CHAPTER V

THE DETERMINATION OF VITAMIN C

1. The International Standard of Reference and the Unit of Vitamin C Activity.
   - A. The need for a simultaneous test on the Standard of reference whenever a determination of vitamin C is made.
   - B. The solution of the International Standard of reference for dosing.
   - C. The general arrangement of the test for a determination of the vitamin C potency of a substance in terms of the International Standard.
2. The Preparation of Guinea-pigs for a Vitamin C Determination.
   - A. Animals suitable for the test.
   - B. Housing of the guinea-pigs.
   - C. Vitamin C-free diets.
3. Criteria for the Measurement of the Response of Guinea-pigs to Doses of Vitamin C.
   - A. Key and Elphick's modification of Höjer's "tooth" method.
   - B. Increase in weight.
   - C. Comparison of the two methods.
4. References.

THE existence of vitamin C as "something in lemon juice and fresh vegetables that prevents scurvy" has been recognised longer than any other vitamin.

Rats do not need to be given vitamin C in their diet. It is, however, found in their livers, even after a long period on a diet deficient in this factor. A recent investigation by Hopkins (1934 and 1935) showed that vitamin C is found in the walls of the small intestine where it is probably synthesised. Thus rats cannot be used for the determination of vitamin C. Guinea-pigs, however, cannot synthesise this factor. When fed on a diet deficient in vitamin C, they begin to lose weight after about 18 days and generally die within 28 days, with macroscopic and microscopic symptoms of scurvy. If, however, they are given vitamin C (ascorbic acid) in sufficient quantities in addition to this diet, they will grow and will not develop scurvy. Hence

guinea-pigs are useful animals for the biological determination of vitamin C.

Since the discovery by Svirbely and Szent-Györgyi (1932) of the antiscorbutic properties of a crystalline substance which has since been named ascorbic acid, much work has been done on the determination of the ascorbic acid content of animal and vegetable tissues by making use of its reducing action on 2, 6-dichlorphenol-indo-phenol (Tillmans, Hirsch and Jackisch, 1932). The reaction is, however, not specific for vitamin C; it is known to be given by certain other substances such as glutathione and adrenaline. For animal tissues Hopkins, Slater and Millikan (1935) have worked out a modification of the method which eliminates the influence of these two substances. Many other modifications have been suggested but they cannot be discussed here.

Kon and Watson (1936) showed that when milk was exposed to light for a short time an oxidised form of ascorbic acid, dehydroascorbic acid, was formed which was biologically active but without action on 2, 6-dichlorphenol-indo-phenol. They proposed reducing this substance with sulphuretted hydrogen before titration. Mack and Tressler (1937) showed that the formation of the oxidised form in vegetable extracts could be prevented by the addition of a strongly ionised acid which destroyed the oxidising enzyme. The reduction with H_2S was thereby eliminated. The concentration of a pure solution of ascorbic acid can be determined by the intensity of its absorption at 245mμ, but in impure solutions the intensity may be modified by other substances. Until the ascorbic acid content of all food substances can be determined by chemical or physical methods the need for a biological method of determination remains.

1. The International Standard of Reference and the Unit of Vitamin C Activity

The Standard of reference recommended for adoption by the Permanent Commission on Biological Standardisation of the League of Nations in 1931 was the freshly expressed juice of the lemon, *Citrus limonum*. The unit of vitamin C activity was 0·1ml. of the fresh juice. The view was accepted that this was less variable in potency than any concentrate available. The opinion as to its constancy of vitamin C content was based on the evidence

that a daily dose of 1·0–1·5ml. of fresh juice was always found to be sufficient to protect guinea-pigs from scurvy for 60 or even 90 days. It was recognised at the time that this was a bad method of judging the constancy of the potency of the juice, for any "complete" animal reaction, other than the death of the animal, is very difficult to judge. Moreover, if a dose of 1·5ml. juice had brought about complete protection, 1·25ml. or 1·0ml. might also have brought it about. It is therefore easily understandable that with "complete protection" as the criterion, early workers on vitamin C were led to believe that lemon juice had a constant vitamin C content. Since the discovery that vitamin C is a substance (ascorbic acid) which can be measured chemically in lemon juice with accuracy, it has been shown that the potency of lemon juice varies greatly. Bacharach, Cook and Smith (1934) in an examination of 15 lemons found extreme values of 0·47 and 0·73mg. per ml., with a mean of 0·64mg. per ml. of the juice.

The Standard of reference recommended for adoption by the Permanent Commission on Biological Standardisation of the League of Nations in 1934 was *l*-ascorbic acid of which Professor Szent-Györgyi was asked to prepare a sample of 500g. Professor W. N. Haworth of the University of Birmingham was invited to co-operate in controlling the purity of the Standard material. The criteria of purity for the International Standard as laid down by the International Conference in 1934 and the findings of Professor Haworth for Szent-Györgyi's sample are :

	I.C.	H.
Melting point	192°C	193°C
Specific rotation in water $[\alpha]_D^{20^\circ}$	+22·4°	+22·6°
(concentration g. per c.c.	2·2	2·34)
Amount in c.c. of N/100 aqueous iodine required by 10mg., the titration being carried out with starch as indicator	11·4	11·4
Absorption spectrum in slightly acid aqueous solution characterised by a single intense band with head at wave length	245mμ	245mμ
Molecular extinction coefficient	10,000	10,000
Ultimate analysis, C	40·9%*	40·95%
,, ,, H	4·6%*	4·6%

Professor Haworth's examination also showed that the Standard preparation agreed with the criteria laid down by the Conference, (1) in showing no appreciable darkening before melting : (2) in being free from ash : (3) in nature of crystals : *i.e.* rectangular plates showing straight extinction with high bi-refringence

* Theoretical.

[$n\alpha$(D-line), 1·47±0·005; $n\beta$(D-line), 1·68±0·005]. No special precautions are required for preserving the activity of the Standard. It may be kept at room temperature in a sealed tube or in an evacuated desiccator. The part needed for dosing must be removed as required each day; no solution must be kept from day to day (see below).

The unit of vitamin C activity recommended for adoption is the vitamin C activity of 0·05mg. *l*-ascorbic acid. This is approximately the average amount of *l*-ascorbic acid in 0·1ml. of fresh

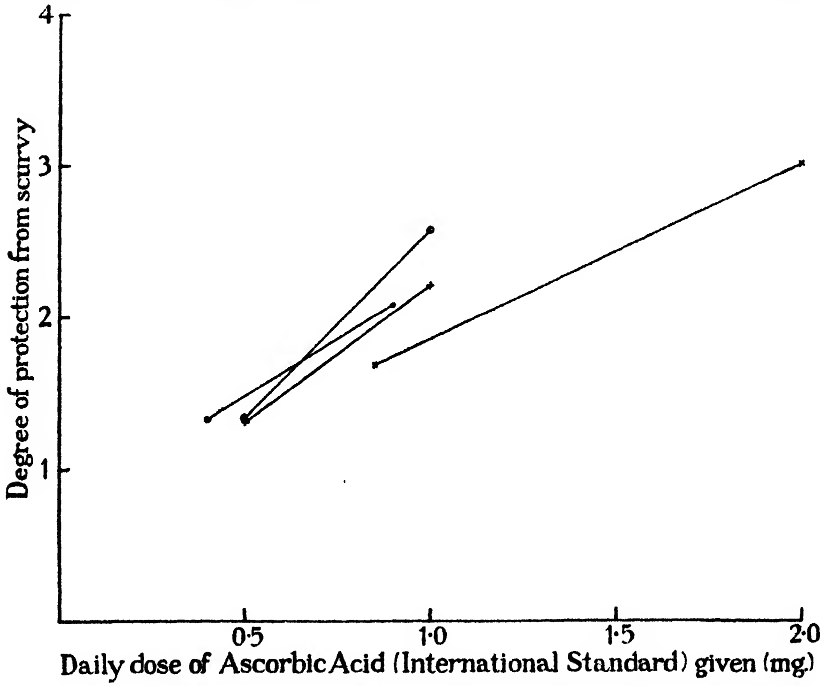

FIG. 12.—Curves of response to doses of vitamin C obtained at different times.

lemon juice. Thus the biological value of the unit of vitamin C activity remained unchanged through the changing of the Standard.

A. The need for a simultaneous test on the Standard of reference whenever a determination of vitamin C is made.

There is only one reference in the literature to the variation in response of guinea-pigs at different times to the same dose of vitamin C. Key and Morgan (1933), using the method of determination developed by Key and Elphick (1931), and testing a

series of five doses ranging from 0·15 to 2·0mg. of ascorbic acid, at three different times, found that the slopes of the curves of response to these doses was the same as the slope of the curve previously obtained by testing a series of doses of orange juice, but that the positions of the curves differed slightly. This was a good indication of a variation in sensitivity of the guinea-pigs used. Further work (unpublished results) from the writer's laboratory has confirmed it (Fig. 12). This result, together with the fact that all investigated animal reactions are known to show variations in intensity from time to time, make it quite evident that whenever a determination of vitamin C is to be made, a simultaneous test of the Standard must be made on similar animals.

B. The solution of the International Standard of reference for dosing.

It is of the utmost importance that the Standard of reference (ascorbic acid) should be dissolved in freshly boiled and cooled, glass-distilled water immediately before it is given to the guinea-pigs each day. A solution of ascorbic acid in water from a copper still will lose much of its vitamin C activity within an hour.

C. The general arrangement of the test for a determination of the vitamin C potency of a substance in terms of the International Standard.

Whatever the criterion chosen for the measurement of the vitamin C potency of a substance, the arrangement of the experiment is the same. Prophylactic (preventive) experiments are usually made for vitamin C though sometimes an economy of labour is effected by giving all the guinea-pigs no supplement of Standard or test substance for 10–14 days at the beginning of the experiment.

A test should be arranged as in similar determinations of other vitamins. Five groups of 5 guinea-pigs each should be used. Two should be given doses of the Standard in the ratio 2 : 1 and three should be given doses of the test substance in the ratio 9 : 3 : 1 if no information is available of the probable potency of the substance. When such information has been obtained, then two doses only, in the ratio 2 : 1 need be tested. Every member of any one group is given the same dose. None of the doses of Standard chosen should be great enough to afford complete protection. The object is to find one dose of the test substance

giving the same result as one dose of the Standard, or possibly two doses of the test substance giving the same results as two doses of the Standard.

The determination of the shape of the curve of response.—Each worker should determine the shape of the curve of response to graded doses (4 or 5) of ascorbic acid using the criterion which he intends to adopt in future assays. Each group of guinea-pigs should consist of at least 5 animals, each animal in a group being given the same dose. Useful daily doses for a fortnight's test are 0·125, 0·25, 0·5, 1·0 and 2·0mg. of ascorbic acid. Results within each group are averaged and the means plotted against the doses given. If the curve proves to be logarithmic then procedure is as described on pp 38–44. The curve of response to doses of vitamin C when histological changes in the teeth were used as the criterion was, however, found to be a straight line by Key and Elphick (1931). The results from such a test require special treatment (see below).

2. The Preparation of Guinea-pigs for a Vitamin C Determination

A. **Animals suitable for the test.**

Young guinea-pigs weighing 200–250g. are used for vitamin C determinations whatever may be the criterion chosen for measuring the effect of the doses of test substance and Standard. It is not necessary that they should be bred in the laboratory in which they are to be used, but it is very essential that they should be obtained from a healthy stock. It is not necessary apparently to have the same proportion of bucks to does in all groups of a test in which the "tooth" method is used for diagnosis, but it is important to have the same proportion of bucks to does in all groups when the "increase in weight" method is used.

B. **Housing of the guinea-pigs.**

The laboratory for animals used in a vitamin C test should be light, airy and warm, for guinea-pigs easily develop pneumonia. Their susceptibility to the disease is a strong argument against long-time methods of testing for vitamin C, for the longer the test period the greater is the chance of intercurrent diseases affecting the results. For long-time tests Bracewell, Hoyle and Zilva (1930) have found it useful to inoculate all animals with a

vaccine consisting of equal numbers of *B. enteritidis*, Gaertner and *B. ærtrycke* before the experiment is begun in order to eliminate intercurrent diseases of intestinal origin.

C. **Diets suitable for vitamin C tests.**

(1) A simple diet which has proved satisfactory for vitamin C work in many laboratories has the following composition :

Bran	45%
Crushed oats	25%
Dried skimmed milk	30%

In addition each guinea-pig is given about 1ml. cod liver oil twice weekly by pipette into the back of its mouth. Tap-water is supplied in an open feeding pot which cannot easily be upset. The food itself is supplied *ad lib.* in a feeding pot with a turned-in rim to prevent spilling. It is not a homogeneous mixture but in practice that seems to be immaterial.

(2) The diet used by Bracewell, Hoyle and Zilva (1930) in their study of the vitamin C content of apples consisted of :

Bran	6 parts by volume.
Barley-meal	2 ,, ,,
Middlings	3 ,, ,,
Fish-meal	1 part by volume.
Crushed oats	4 parts by volume.

This was given *ad lib.* in addition to 40–60ml. of autoclaved milk made up from a dried full-cream powder. No cod liver oil was given with the diet, the vitamin A needed by the pigs being supplied by the fish-meal and milk.

3. Criteria for the Measurement of the Response of Guinea-pigs to Doses of Vitamin C

(*a*) *Onset of scurvy.*—The symptoms of scurvy noticeable in the living guinea-pig are stiffness of the joints, particularly of the hind legs, a tendency to sit crouched up and, later, the "face-ache" position of the head, cessation of growth and loss of weight. Death of the animal will follow within 28 days of the withholding of vitamin C from the diet. The symptoms are more easily recognised by the trained worker than described. If guinea-pigs are killed when they first appear to be developing scurvy, slight hæmorrhages are found at the joints, particularly at the knees, the ribs are beaded and the gums are swollen and may have hæmorrhages also. These macroscopic lesions may

easily be classified as slight, severe, very severe, etc., and a rough comparison can be made between the groups of animals that have received different doses.

(*b*) *Histological changes in the teeth.*—Zilva and Wells (1919) first showed that guinea-pigs fed on a scorbutic diet developed changes in the structure of the teeth. Höjer (1924, 1926) examined these changes and claimed that they could be measured so that a quantitative determination of vitamin C could be based on them. Goettsch (1928) thought that Höjer had claimed too great a degree of accuracy for his method, and Key and Elphick

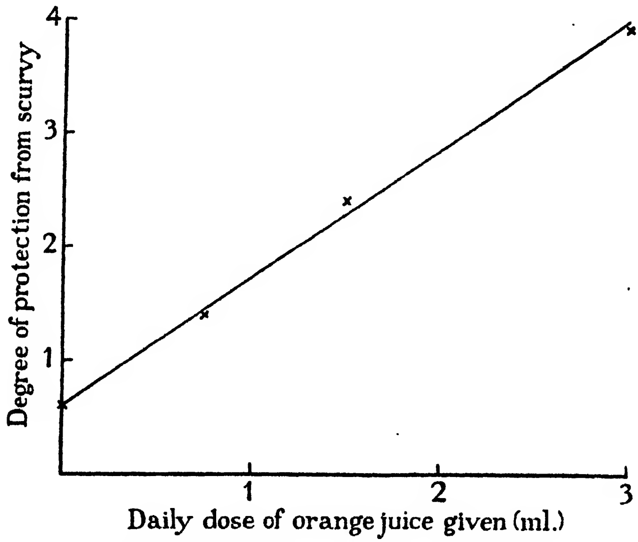

FIG. 13.—Curve of response relating the amount of protection from scurvy and the amount of vitamin C (as orange juice) given. (Constructed by Key and Elphick.)

(1931) drew up a scale of disordered teeth (Figs. 15–19) (based partly on Goettsch's work) and a curve of response (Fig. 13) to graded doses of vitamin C, by means of which more accurate comparisons could be made than had hitherto been possible. The accuracy obtainable by the method was examined by Coward (1934), and found to be very similar to that obtainable in other vitamin tests.

(*c*) *Increase in weight of the guinea-pig.*—Zilva has used the increase in weight in 60–90 days of guinea-pigs given a scorbutic diet plus the substance containing the antiscorbutic factor as a

means of comparing the vitamin C potency of different substances. He states that guinea-pigs fed on his scorbutic diet alone will die within 28 days, and that the increase in weight of other guinea-pigs given the vitamin is proportional to the dose of vitamin given. In comparing the vitamin C potency of different kinds of apples, and of apples of the same kind grown under different conditions, or stored under different conditions, he has equated the doses that gave approximately the same rates of growth in his animals.

Coward and Kassner (1936) modified this method in two respects: (*a*) a simultaneous test on the International Standard of reference was made; (*b*) the period of the test was shortened to 6 weeks.

A. **Key and Elphick's modification of Höjer's "tooth" method.**

Höjer claimed that he was able to distinguish between doses of vitamin C differing by as little as 10%, by examination of the teeth of the guinea-pigs used.

When Goettsch (1928) tried this method, using a 2 weeks' test period, she found that two doses differing by as much as 300% might bring about equal responses in 2 animals. This is no greater than the variation found in most other animal reactions, but it means that very large numbers of animals would have to be used and the results averaged to distinguish between doses differing only by 10%.

Key and Elphick (1931) showed that a graded response to graded doses of vitamin C could be obtained by using groups of about 15 animals for each dose tested. The results obtained from the different pigs of a group were averaged by comparing the tooth section of each pig with an arbitrary scale which they had drawn up depicting teeth in different stages of the disorder brought about by a lack of vitamin C. (Figs. 15–19). The use of this method then follows the general plan of a vitamin determination.

(*a*) *Dosing the guinea-pigs.*—Whenever possible, the dose of substance to be tested should be given directly into the guinea-pig's mouth. When the substance is a fruit juice this can easily be done from a pipette and generally fruit juices are so potent that the dose needed for the test is small and can be given quickly. The guinea-pig is allowed to sit comfortably on a table, its head firmly held in one position and the end of the pipette placed far

back in its mouth. No attempt should be made to make the guinea-pig swallow his dose more quickly than he wishes to, or part of the dose may be lost. If the volume of liquid to be given is large it may be given in two or three parts at intervals of a few hours. This plan is useful when milk has to be tested, for the vitamin C value of milk is low.

The International Standard is easily handled. It is soluble in water and the required weight can be given to a guinea-pig conveniently in a volume of 2ml. water. The precaution mentioned in the description of the Standard may be repeated here. The solution should be made up immediately before being given to the pigs and only freshly prepared, glass-distilled water should be used.

Doses may be given daily for 14 days, with double doses on Saturday and none on Sunday.

(*b*) *Preparation of the teeth for examination.*—At the end of the 14 days' period the guinea-pigs are killed and examined for any macroscopic symptoms of scurvy. It is unusual to find any symptoms as they seldom develop in so short a time as 14 days of feeding without vitamin C. The absence of these lesions is a good indication that the animals were not in a scorbutic state at the beginning of the experiment.

The lower jaw-bone is removed and freed as completely as possible from adhering muscle and skin. It may be divided into two by cutting between the incisors. The parts are placed in decalcifying solution until soft enough to cut with a scalpel. The decalcifying solution consists of:

Concentrated hydrochloric acid	4ml.
Glacial acetic acid	3ml.
Chloroform	10ml.
Water	10ml.
Alcohol 97%	73ml.

Each jaw-bone requires about 100ml. of the solution for complete decalcification. The part required for examination is the root of the incisor in the region of the bend of the jaw just in front of the first molar (Fig. 14). The unneeded parts can be cut away when soft enough, to hasten the decalcification of the rest. The small piece of bone is preserved in 80% alcohol till required for cutting. It is important to cut the section from the same part of the jaw-bone each time, for Fish and Harris (1934) have shown that different parts of the jaw-bone are affected in different degrees.

The details of the preparation of the section are taken from Key's appendix in Goettsch's paper (1928).

(i) *Embedding in gelatin.*—The alcohol is gradually diluted by adding a small quantity of distilled water about every 10 minutes. At the end of half an hour the tooth is transferred to distilled water. This process prevents the tearing of the tissues by sudden changes of surface tension. Meanwhile a test-tube containing a 20% solution of gelatin is heated in a waterbath to 40° C. The piece of tooth is then dropped into the gelatin and the bath is maintained at 35°–40° C. for half an hour. At the end of that time the gelatin, carrying with it the piece of tooth, is poured into a small, shallow dish which has been previously wetted with water. The piece of tooth is arranged with the cut-surface showing the base of the incisor parallel to the surface of the gelatin. The liquid is then left to set. When it is cold a small cube of gelatin,

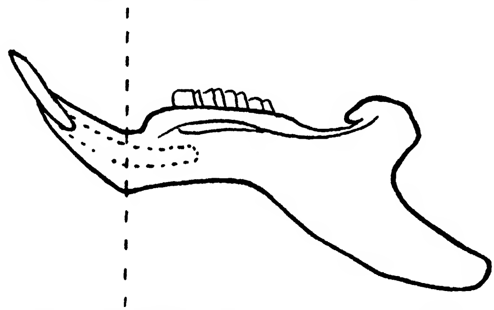

FIG. 14.—Jaw-bone of guinea-pig. Dotted line, plane of section.

containing the tooth, is cut out and placed in a test-tube containing 4% solution of formaldehyde, where it is left for 24 hours to harden.

(ii) *Cutting and mounting the sections.*—Two microscope slides for each tooth are prepared by carefully washing with soap and hot water; then one side of each is coated with a thin layer of Kaiser's glycerin jelly which is prepared as follows:

Forty grams of gelatin are soaked in 210ml. water for 2 hours, then heated with 250ml. glycerine and 5gm. carbolic acid for 10–15 minutes. The solution is filtered through ordinary filter paper at 54° C. On cooling it sets to a jelly. Before use, this is heated on a water-bath until liquid. A very small quantity is spread over each slide with a glass rod. The slides are allowed to stand for 3 hours so that the jelly may set.

The gelatin cube containing the tooth is transferred from the formaldehyde solution to water and left for about half an hour.

The tooth is then cut with a freezing microtome, adjusted to give sections 15μ thick. The sections are placed in a dish of water. Six or eight of them are mounted on each of the prepared slides by means of a glass needle. A clean cigarette paper, supported at the ends by two or three folded papers, is carefully placed over the sections. It is then covered with a thick pad of papers and firmly pressed down. When the papers are removed, the sections remain embedded in the thin layer of gelatin.

(iii) *Staining*.—Two sets of stains are used.

1. Hansen's hæmatoxylin and Eosin.—To prepare Hansen's hæmatoxylin, three solutions are made as follows :

(*a*)	Hæmatoxylin crystals	1gm.
	Absolute alcohol	10ml.
(*b*)	Potash (or ammonium) alum	20gm.
	Distilled water	200ml.
(*c*)	Potassium permanganate	1gm.
	Distilled water	16ml.

The following day solutions (*a*) and (*b*) are mixed, then 3ml. of solution (*c*) are added. The liquid is boiled for 1 minute with continuous stirring, then quickly cooled by placing the vessel containing the liquid in a basin of cold water.

One of the two slides, prepared as above, is placed in this solution for 6–8 minutes. It is then washed in running water for 10 minutes and afterwards left in distilled water for any convenient length of time.

The excess gelatin is wiped off at this stage as it is difficult to clean the slide later.

The process is completed by immersing the slide in :

(*a*) 0·5% aqueous solution of eosin for 1–2 minutes,
(*b*) 96% alcohol for 1½ minutes,
(*c*) fresh 96% alcohol for a further 1½ minutes,
(*d*) absolute alcohol for 3 minutes,
(*e*) xylol for about 2 minutes,
(*f*) fresh xylol for a further 5 minutes.

The cover slips are attached by means of 2 or 3 drops of a solution of Canada balsam in xylol.

2. Hansen's Tri-oxyhæmatin and Connective Tissue Stain.—To prepare Hansen's tri-oxyhæmatin, two solutions are made up as follows :

(*a*)	Ferric alum	10gm.
	Distilled water	150ml.
(*b*)	Hæmatoxylin	1·5gm.
	Warm distilled water	75ml.

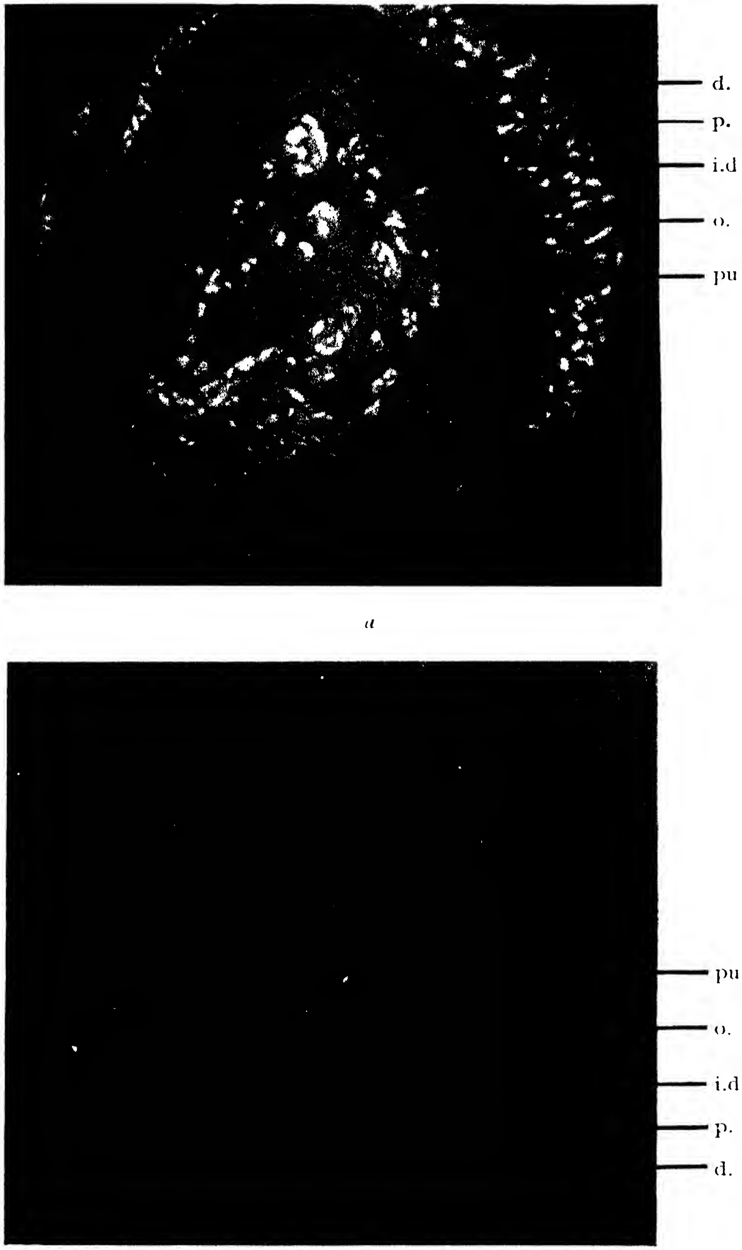

FIG. 15, *a* and *b*.—Amount of protection, o. The amount of protection afforded by vitamin C against histological changes in the structure of the incisors of guinea-pigs brought about in 14 days.

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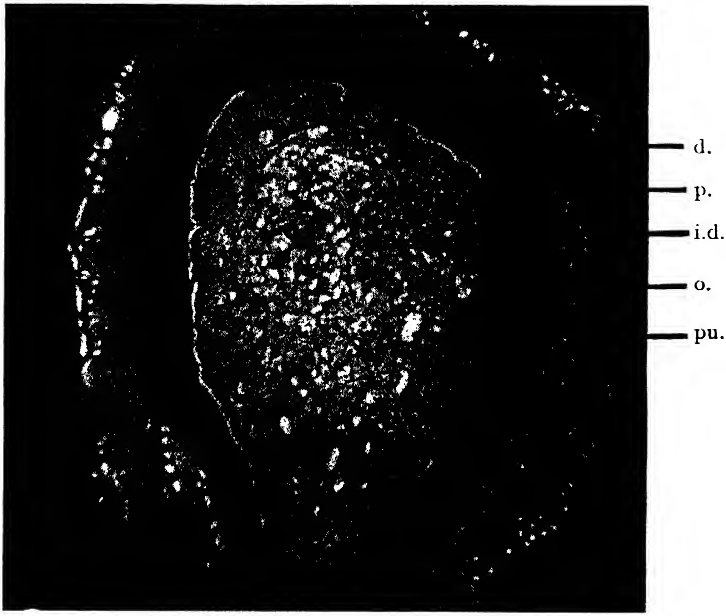
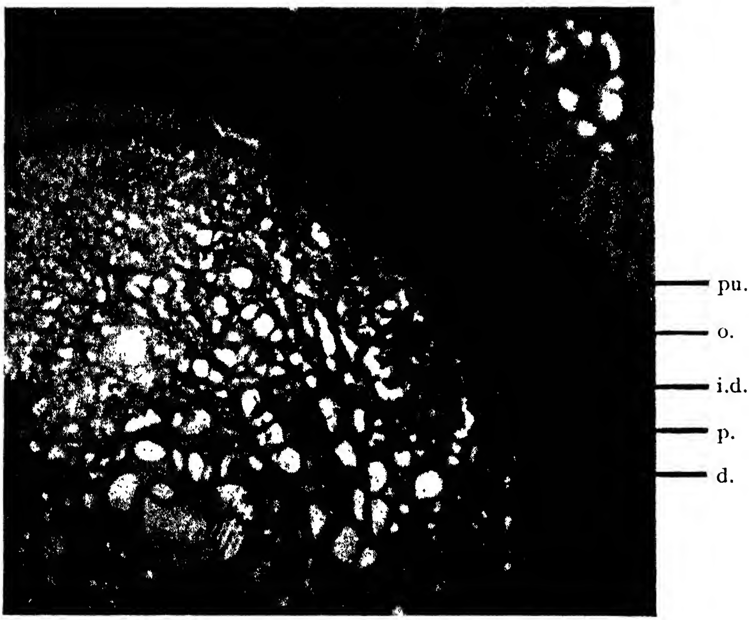
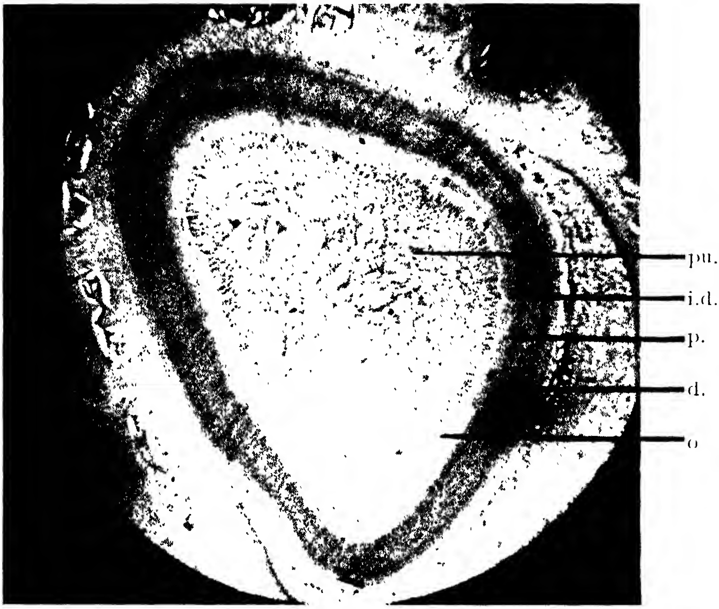


a

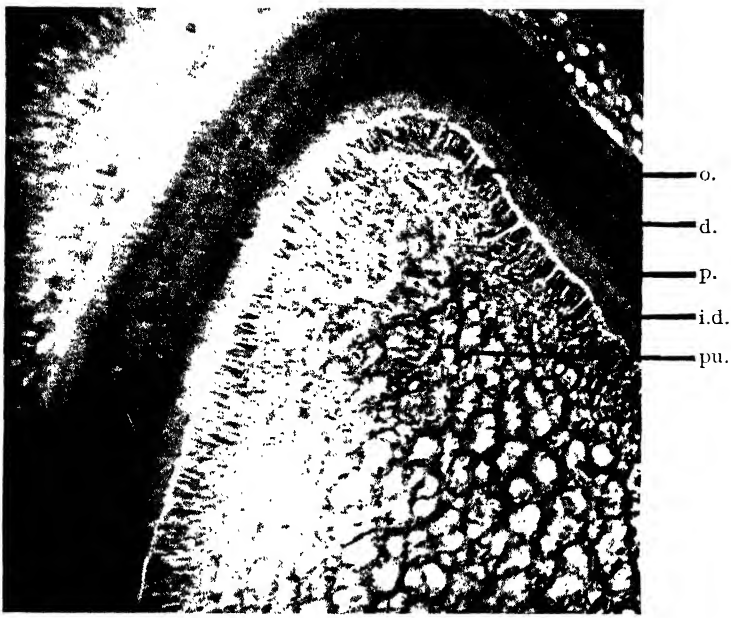


b

FIG. 16, *a* and *b*.—Amount of protection, 1.

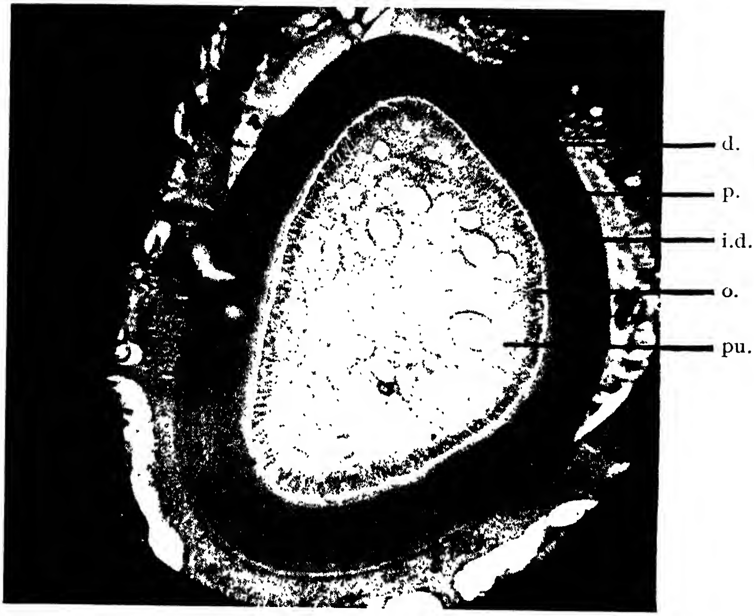


a

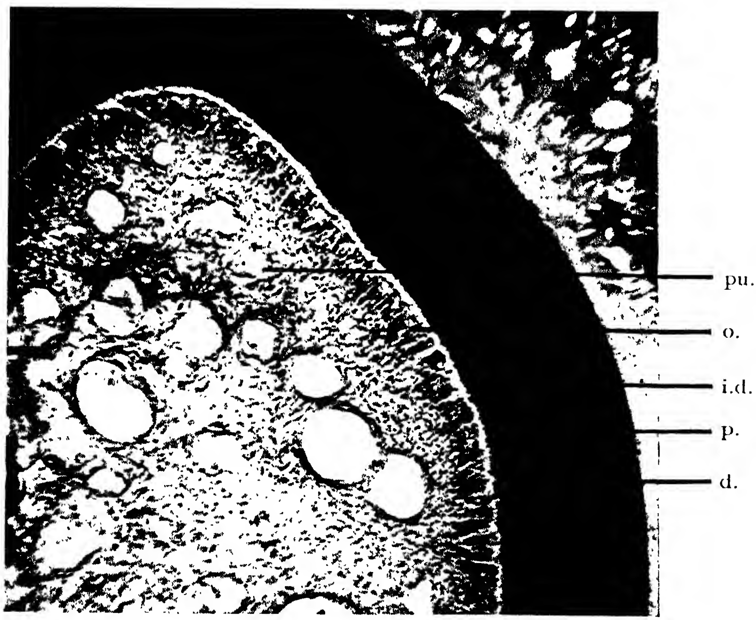


b

FIG. 17, *a* and *b*.—Amount of protection, 2.

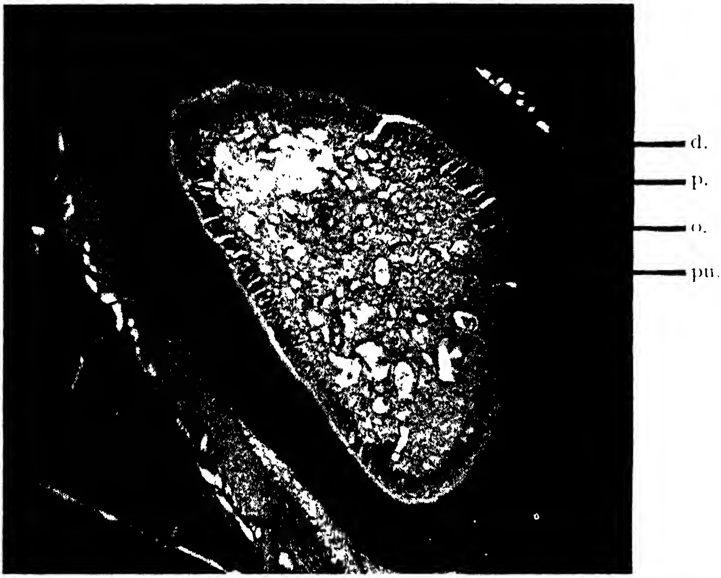


a



b

FIG. 18. *a* and *b*.—Amount of protection. 3.



a

b

FIG. 19, *a* and *b*.—Amount of protection, 4.

Solution (*a*) is poured into solution (*b*) with stirring. The mixture is heated and allowed to boil for half a minute. When cold, the liquid is dark brown with a slight precipitate. It is filtered before use.

The second slide on which sections have been mounted is left in this solution for 1 hour, then washed for 10 minutes in running tap water. The excess gelatin is wiped off and the slide placed in distilled water.

Counter-staining is effected with Hansen's connective tissue stain which is prepared as follows:

100ml. of saturated aqueous solution of picric acid is mixed with 5ml. of 2% aqueous solution of acid fuchsin. This mixture is kept as a stock solution and 7 drops of 1% acetic acid solution are added just before use.

The slide is kept in this dye for 20–30 minutes, then rinsed in acidulated water (30ml. distilled water and 20 drops of 1% acetic acid solution) for a few seconds. The sections are dehydrated in alcohol and cleared in xylol and the cover-slips are attached with Canada balsam.

(*c*) *Working out the result.*—In order to average the results given by the different members of any group of guinea-pigs, all of which have been given the same daily dose, each tooth section must be compared with a scale consisting of a series of sections showing graded degrees of protection which have been given numerical values corresponding to the degree of protection shown. Such a scale (Figs. 15–19) was drawn up by Key and Elphick (1931). The cross-section of a normal tooth consists of pulp in the middle surrounded by a layer of long parallel odontoblasts, a narrow predentine and a wider dentine. Fine Tomes' canals run from the odontoblasts through the predentine and dentine. In a scorbutic tooth, the odontoblast layer is disorganised, the predentine is calcified and an irregular layer of bone is formed between the predentine and the odontoblasts. The Tomes' canals are fewer and are only found radiating from the predentine outwards. The extent of the disorder of each part varies with the smallness of the dose of vitamin C given, but with a particular dose, the disorder of all parts is not always greater than the disorder of all parts in a pig given a larger dose. Thus an average value for a tooth has to be assigned from a consideration of all parts that may be disordered. Key and Elphick found the scheme in Table IV useful for evaluating the amount of protection from

scurvy afforded by a dose of vitamin C, the figure 0 being given to the severest form of scurvy developed in 2 weeks by feeding pigs on a completely scorbutic diet, and the figure 4 being given to a state of complete protection from scurvy.

TABLE IV

SCHEME FOR ASSESSING THE SEVERITY OF SCURVY DEVELOPED IN GUINEA-PIGS IN 14 DAYS WHEN GIVEN A DIET PARTLY OR WHOLLY DEFICIENT IN VITAMIN C

Odontoblasts.	Inner dentine.	Predentine.	Tomes' canals.	Degree of protection from scurvy.
Disorganised	Wide, with projections into pulp	Calcified	Only in outer dentine	0
Completely disorganised in places, but some parallel formation	Narrow and irregular	Calcified	Only or mostly in outer dentine	1
All parallel but becoming disorganised near pulp	Narrow	Calcified or partly calcified	Mostly in outer dentine or cross inner dentine from odontoblasts	2
Long and parallel but becoming disorganised near pulp	Absent or mere rim	Not calcified	Cross dentine from odontoblasts	3
Long and parallel	Absent	Not calcified	Cross dentine from odontoblasts	4

Thus by using a scheme such as this a numerical value can be given to each tooth. The values for all the guinea-pigs in a group can be averaged and the averages of the different groups, given different doses, can be compared. Whatever may have been the arrangement of doses chosen, this method of evaluating results may be used.

Example.—A certain concentrate was examined in the following way: 30 guinea-pigs were divided into five groups of 6 animals each. All the pigs in any one group were given the same dose; four of the different groups were given daily doses of 0·4mg. ascorbic acid (not the International Standard), 0·8mg. ascorbic acid, 0·75ml. diluted concentrate and 1·5ml. diluted concentrate respectively, and the fifth group was given no dose.

The result of the examination of the teeth and evaluation according to the scheme in Table IV was summarised as follows :

Dose.	Protection afforded to each guinea-pig.						Average protection afforded to the group.
0·4mg. ascorbic acid	1·5	0·5	2·0	1·0	1·0	2·0	1·33
0·8mg. ascorbic acid	2·0	3·5	1·5	2·5	1·5	1·5	2·08
0·75ml. diluted concentrate	1·5	0·5	1·0	1·0	1·5	1·0	1·08
1·5ml. diluted concentrate	3·0	1·0	3·5	2·5	3·5	3·0	2·75
No dose	1·5	1·5	1·0	0·0	0·0	1·0	0·83

(i) By a simple comparison of the amount of protection afforded by each dose given to the different groups of guinea-pigs, it is evident that :

(*a*) 0·4mg. ascorbic acid has afforded a little more protection than 0·75ml. of the diluted concentrate ; thus 0·75ml. of the diluted concentrate appears to be a little less potent than 0·4mg. ascorbic acid.

(*b*) 0·8mg. ascorbic acid has afforded a little less protection than 1·5ml. of the diluted concentrate ; thus 1·5ml. of the diluted concentrate appears to be a little more potent than 0·8mg. ascorbic acid.

From the two comparisons it may be concluded that 1·0ml. of the diluted concentrate is equivalent to about 0·5mg. of the ascorbic acid with which it was compared. (*Note.*—As this was not the actual sample of ascorbic acid which is in use as the International Standard, the result cannot be expressed in International Units.)

The figures given in column 2 of the list of results of this experiment indicate the amount of variation that may be expected between different animals.

(ii) *Calculation of the result.*—The curve of response relating amount of protection from scurvy to dose of vitamin C given is a straight line, not logarithmic. Therefore, if lines of average slope were drawn through the "mid-points" of two curves for Standard and test substance, the horizontal distance between them would certainly be the same at all levels but it would be a difference in dose given, not a difference in the log. of the dose given. Hence this difference would be a different percentage of the potency of the Standard according to the level at which it

was read. If, however, the log. of the amount of protection is plotted against the log. of the dose given, a straight line is again obtained, but the horizontal distance between two such lines would be the log. of the ration of the potencies of the two substances. The calculation of the result recorded above can then be made as follows :

Average protection.	Log. of average protection.	Dose given.	Log. of dose given.
1·33	0·12385	As. ac. 0·4mg.	$\bar{1}$·60206
2·08	0·31806	,, 0·8mg.	$\bar{1}$·90309
	Diff. = 0·19421		Diff. = 0·30103
1·08	0·03342	Dil. conc. 0·75ml.	$\bar{1}$·87506
2·75	0·43933	,, 1·5ml.	0·17609
	Diff. = 0·40591		Diff. = 0·30103

$$\text{The average slope is } \frac{0{\cdot}19421+0{\cdot}40591}{2\times 0{\cdot}30103}=0{\cdot}9968$$
$$=1{\cdot}0 \text{ (say).}$$

The point with average abcissa and ordinate on the curve for ascorbic acid is $\bar{1}$·75258, 0·22096. The point with average abcissa and ordinate on the curve for diluted concentrate is 0·02558, 0·23638. The curve with average slope, 0·9968, through the "mid-point" of the curve for the ascorbic acid is

$$\frac{y-0{\cdot}22096}{x-\bar{1}{\cdot}75258}=0{\cdot}9968$$

therefore $$y=0{\cdot}9968x+0{\cdot}46759$$

The curve with average slope, 0·9968, through the "mid-point" of the curve for the diluted concentrate is

$$\frac{y-0{\cdot}23638}{x-0{\cdot}02558}=0{\cdot}9968$$

therefore $$y=0{\cdot}9968x+0{\cdot}21088$$

The horizontal distance between these two lines is

$$\frac{0{\cdot}21088-0{\cdot}46759}{0{\cdot}9968}=-0{\cdot}2575=\bar{1}{\cdot}7425$$
$$=\log.\ 0{\cdot}55272$$

Therefore the diluted concentrate contains about 0·55mg. ascorbic acid per ml.

The student is recommended to work out the above result using only 2 figures of decimals. (See also Finney, 1945.)

B. **Increase in weight.**

The influence of vitamin C on the increase in weight of guinea-pigs has been established by Bracewell, Hoyle and Zilva (1930). In their report on the "Anti-scurvy Vitamin in Apples" they say that in assessing the biological results quantitatively, one has to consider several independent factors, namely, duration of test period, cause of death, first appearance of scorbutic symptoms, degree of scurvy at autopsy, and growth. They have published thirty-three charts of growth curves of animals. Each chart represents the test on 10–24 guinea-pigs in three or four groups, the groups having been given different doses of one sample of apple and all the pigs in any one group having been given the same dose. The curves of growth of the animals of any one group are drawn in the same diagram. Thus it is easy to see how far the different animals have varied in their response to a particular dose of a particular kind of apple and also to get a general idea of how far different groups of animals varied in their response to different doses of the same sample of apple. The test was carried on for 60–90 days. The diet used has already been described in this chapter. The doses tested were generally 3, 5, 10 and 20g. respectively. No simultaneous tests on a standard of reference were made; comparisons between different samples were made from results obtained at the same or at different times from the animal reactions. Thus no consideration was given to possible differences in sensitivity of the guinea-pigs at different times and no allowance was made for the possibility.

The charts, however, demonstrate the following points:

(*a*) Given a small dose of vitamin C (in apple), most guinea-pigs will grow for about 3 weeks but will then cease to grow and may lose weight and die.

(*b*) Given a rather larger dose, the same initial increase in weight will be observed, and it may be followed by a long period of neither increase nor decrease in weight.

(*c*) Given a larger dose still, increase in weight will continue till the pigs reach a weight of 500g. or more.

If vertical lines are drawn through the points representing the end of 4 weeks of these tests, the following observations may be made:

(*a*) The variation in result in all the groups is very great.

(*b*) There seem to be three types of results distinguishable at this stage:

(1) Loss of weight.

(2) Maintenance of weight.

(3) Increase in weight, which is curiously about the same rate in all the groups that show increase in weight at all in each chart.

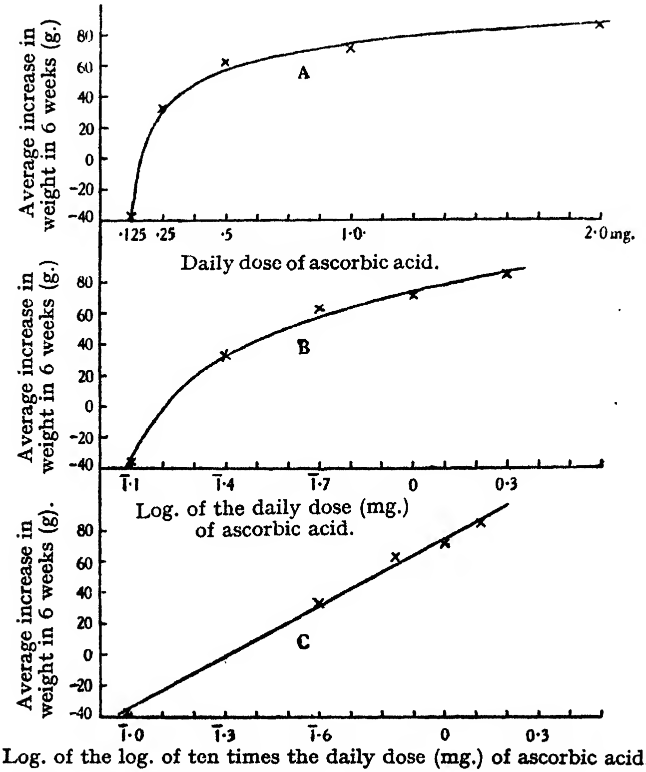


FIG. 20.—The mean increases in weight of groups of guinea-pigs given graded doses of ascorbic acid daily for 6 weeks plotted against

(A) x=the dose of ascorbic acid, equation $y=74\cdot3+108\cdot2$ log. (log. $10x$);

(B) x=the log. of the dose of ascorbic acid, equation $y=74\cdot3+108\cdot2$ log. $(x+1)$;

(C) x=the log. of the log. of ten times the dose of ascorbic acid, equation $y=74\cdot3+108\cdot2x$;

to show how the equation of the curve of response was obtained.

The difference between loss of weight and maintenance of weight is attained by doubling the dose. The difference between maintenance of weight and increase in weight is attained by doubling the dose. Redoubling the dose again does not increase the rate of growth any further.

This indicates that the curve of response relating increase in weight of guinea-pigs to dose of vitamin C given is very steep which indicates that this should be an accurate method of determination of vitamin C.

Coward and Kassner (1936), recognising the simplicity of the "increase in weight" method, constructed a curve of response relating increase in weight of guinea-pigs in 6 weeks to dose of vitamin C given. As was foreseen from Zilva's results the curve was a very steep one (Fig. 20), but they found that the accuracy obtainable by its use is about the same as that obtainable in the "tooth" method.

A curve drawn freehand through the points plotted on graph paper did not appear to be logarithmic in shape but nevertheless the average increases in weight were plotted against the logs. of the doses given. The curve obtained (Fig. 20, B) was not a straight line, but looked to be itself logarithmic in shape. To test this the average increases in weight were then plotted against the logs. of the (log. +1)'s of the doses given, *i.e.* against the logs. of 10 times the doses given (since there are no logs. of minus quantities). This gave a straight line (Fig. 20, C) of the form $y=74{\cdot}3+108{\cdot}2x$ in which $x=$ log. (log. of 10 times the dose).

The curve (Fig. 20, A) was drawn from the following data :

Average increase in weight in 6 weeks, g.	Dose of ascorbic acid (Inter. St.) given daily, mg.	Log. of dose of ascorbic acid given.	Log. dose + 1·0, *i.e.* log. of 10 times the dose.	Log. (log. dose + 1), *i.e.* log. (log. of 10 times the dose).
−37·3	0·125	$\bar{1}$·09691	·09691	$\bar{2}$·98637
+33·0	0·25	$\bar{1}$·39794	·39794	$\bar{1}$·59982
+62·8	0·5	$\bar{1}$·69897	·69897	$\bar{1}$·84446
+71·2	1·0	0·0	1·0	0·0
+84·2	2·0	0·30103	1·30103	0·11427

Doses of a fruit juice were tested at the same time and gave the following results :

Average increase in weight in 6 weeks, g.	Dose of fruit juice given daily, ml.
−77·7	0·25
−25·5	0·5
+43·2	1·0

These figures admit of the same treatment as those for the ascorbic acid and give the equation $y=34{\cdot}6+294{\cdot}25x$ in which

$x=$ log. (log. of 10 times the dose), but this is a much steeper curve than that for ascorbic acid.

The average slope of the two curves allowing for the total number of animals used in testing the two substances and the numbers of animals in each group is

$$\frac{108{\cdot}2\times38+294{\cdot}25\times21}{59}=174{\cdot}4$$

The "mid-point" in the curve for ascorbic acid is $\bar{1}{\cdot}70898$, 42·8. The "mid-point" in the curve for the fruit juice is $\bar{1}{\cdot}81476$, $-19{\cdot}9$. The curve with average slope, 174·4 through the "mid-point" of the ascorbic acid curve is

$$\frac{y-42{\cdot}8}{x-\bar{1}{\cdot}709}=174{\cdot}4$$

therefore $$y=174{\cdot}4x+93{\cdot}6$$

The curve with average slope, 174·4 through the "mid-point" of the fruit juice curve is

$$\frac{y-(-19{\cdot}9)}{x-\bar{1}{\cdot}81476}=174{\cdot}4$$

therefore $$y=174{\cdot}4x+12{\cdot}4$$

The horizontal distance between these two parallel lines is

$$\frac{12{\cdot}4-93{\cdot}6}{174{\cdot}4}=\frac{-81{\cdot}2}{174{\cdot}4}=-0{\cdot}4656=\bar{1}{\cdot}5344$$
$$=\text{log. } 0{\cdot}34230$$

0·3423 is the log. of 10 times the difference between doses given.

Therefore $\bar{1}{\cdot}3423$ is the log. of the difference between doses given.
$$=\text{log. } 0{\cdot}21994$$

Therefore the juice has 0·22 of the potency of the ascorbic acid.

Therefore it contains about 0·22mg. ascorbic acid per ml.

C. Comparison of the two methods.

The great advantages of the "tooth" method over the "increase in weight" method are (1) that it is specific for vitamin C, and (2) that the time required for the "tooth" method is shorter than the time required for the "increase in weight" method.

The advantages of the "increase in weight" method over the "tooth" method are (1) that it requires no preparation of sections for histological examination, and (2) that the assessment of the response is made from the weight of the animal.

The two methods give results of approximately equal accuracy. Thus the choice of method must depend on the skill available for the carrying out of the determination. If histological technique can be used, there can be no doubt that the "tooth" method, being specific for vitamin C, is far superior to the "increase in weight" method which is not specific for vitamin C.

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CHAPTER VI

THE DETERMINATION OF VITAMIN D

VITAMIN D promotes the calcification of bone and the formation of hard dentine and enamel in teeth. When the ratio of calcium to phosphorus in the diet is unity less vitamin D is necessary than when the ratio of these elements is greater or less than unity. A recent summary of the evidence on this point, together with fresh experimental evidence, has been made, in English, by Querido (1935). The ratio Ca : P=4 : 1 generally produces rickets in rats which have low reserves of vitamin D, and hence it is useful for experimental purposes. The existence of more forms than one of vitamin D and their use by different species of animals is discussed on p. 137.

1. The International Standard of Reference

The International Standard of Reference for vitamin D is a solution of irradiated ergosterol in olive oil kept at the National

Institute for Medical Research, London. (It is hoped, however, to replace it shortly by a preparation of vitamin D_3 from irradiated 7-dehydrocholesterol.)

The International unit of vitamin D is the activity of 1mg. of the International Standard of Reference.

This is the unit which was recommended for adoption by the Permanent Commission for Biological Standards of the League of Nations in 1931. It was not modified in any way when the standards were reconsidered in 1934. It is the unit that the Medical Research Council of Great Britain had adopted in 1930 and it is also the unit that the Pharmaceutical Society of Great Britain had used since 1927. The conference in 1934 further recommended that when the Standard solution became exhausted or it should become unsatisfactory, it should be replaced by an equivalent solution of pure crystalline vitamin D in olive oil, of such strength that 1 milligram contains 0·025 microgram (0·025μg.) of crystalline vitamin D.

The properties of crystalline vitamin D (calciferol or vitamin D_2, $C_{28}H_{43}OH$) are as follows :

(*a*) colourless acicular crystals, odourless ; melting point, 114·5 to 117° C. (open capillary).

(*b*) Specific rotation

in alcohol $[\alpha]_D^{20°} = +101°$ to $102·5°$; $[\alpha]_{5461}^{20°} = +119°$ to $+122°$.

in chloroform $[\alpha]_D^{20°} = +52°$; $[\alpha]_{5461}^{20°} = +62°$.

(*c*) Absorption spectrum : - in alcohol or other suitable non-absorbing solvent, a smooth curve with a maximum at 265mμ ($E_{1cm.}^{1\%}$ 470 to 485).

It is necessary to keep the Standard solution at 0° C. or below and withdraw it from the cold store for as short a time as possible whenever it is needed for making a dilution for tests.

A. The need for a simultaneous test on the Standard of reference whenever a determination of vitamin D is made.

The severity of the rachitic condition developed in young rats by feeding them on a diet which is deficient in vitamin D and

which has a high-calcium, low-phosphorus content depends on many factors, the chief being:

(i) the rats' reserves of vitamin D, and
(ii) the length of time during which the rachitogenic diet is given to the rats, *i.e.* the preparatory period.

The more severe the rachitic condition of the rats the less is their response to a particular dose of vitamin D. To gain some idea of the extent of the influence of the length of the preparatory period on the amount of healing produced by a particular dose of vitamin D, an experiment was carried out in the writer's laboratory. Five litters of rats consisting of 6, 8, 4, 4 and 8 rats respectively were divided into two groups, one-half of the animals of each litter being assigned to one group and the other half to the other group. The preparatory period of one group was 21 days and of the other group 27 days. Each animal was given 5 units of vitamin D at the end of its preparatory period, and kept on test without further supplement for 10 days. Healing was assessed by the "line" test as carried out in that laboratory (described later in this chapter). The average healing of the rats which were given the dose of vitamin D after 21 days' preparation was 3·2 (on a scale in which 6 represented complete healing) and the average healing of the rats which were given the dose of vitamin D after 27 days' preparation was 2·4. Thus the influence of the length of the preparatory period on the amount of healing brought about by a dose of vitamin D is appreciable.

It must never be assumed that rats which have apparently received the same treatment have developed the same sensitivity to vitamin D, and a simultaneous test must be made on the Standard of reference whenever the vitamin D content of a substance is determined. The fluctuations in the response of the rats from one colony to the same dose of vitamin D (International Standard) have been shown by Bourdillon, Bruce, Fischmann and Webster (1931) (Fig. 21), and a similar result from another colony has been shown by Coward and Key (1933). All the rats had been subjected to the same treatment as far as could be determined and yet the healing brought about by the same dose of the Standard at different times varied so much that at one time it was more than twice as great as at another time. The figures were averages of large numbers of rats and the fluctuations were gradual but not, apparently, seasonal. Thus the differences in response

were not merely the differences one expects to get between individual rats, but were apparently due to some other influence over which the workers had no control.

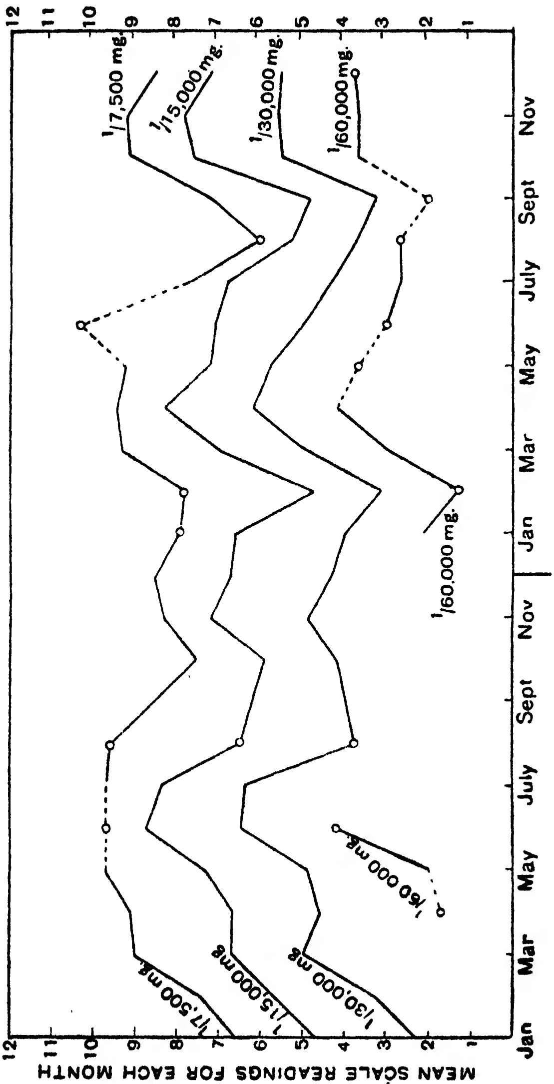


Fig. 21.—Variations in degree of healing caused by constant doses of vitamin D (irradiated ergosterol) in successive months. The average number of rats for each point plotted is 19. Points for which less than 10 rats were available are surrounded by circles. The dotted portions of the curve refer to points for which only 3, or 4, rats were available. (Bruce, Bourdillon, Fischmann and Webster, 1931). No later records are available.

It is evident that a sample of cod liver oil examined simply by its influence on a group of animals would produce different amounts of healing at different times in the same laboratory, and

its vitamin D content would therefore appear to vary from time to time. It might be expected that this difficulty could be overcome by examining the rats' bones by X-ray just before dosing with the cod liver oil and always using only those rats which appeared to have rickets of equal severity. But that was exactly the procedure followed by Bourdillon *et al.* and yet they obtained the fluctuations described. In Table V may be seen examples of the variation in average response of groups of rats obtained in the writer's laboratory and the gradual fluctuations of the response month by month throughout several years.

For many years workers have taken the precaution of dividing litters of rats equally between standard and test substance, being guided partly by commonsense and partly by experience. In 1941 Coward and Kassner so arranged a comparison of two samples of irradiated ergosterol that they obtained convincing evidence (*a*) that the variation between litters in response to vitamin D was real and greater than that between rats of the same litter and (*b*) that the initial weight of the rat had some influence on the amount of healing produced by a given dose of vitamin D. (See Chapter XII.)

Thus the only possible way to use the Standard of reference for vitamin D is to divide each litter of rats available for a determination into two groups as nearly alike as possible and to use one group for dosing with the International Standard and the other group for dosing with the substance whose vitamin D potency is to be determined.

It follows from the above that it is completely wrong to examine the effect of a dose or of several doses of the International Standard on the rats of any colony, however uniform it may appear to be, and then to compare results obtained subsequently with the results previously obtained on the International Standard. This does *not* give a result in terms of the International Standard. That can only be obtained by making a simultaneous test on the Standard whenever a determination of the vitamin potency of a substance is made.

B. The dilution of the Standard of reference for dosing.

Since the International Standard of reference is a solution of irradiated ergosterol in olive oil of such a strength that 1mg. of the solution contains 1 International unit of activity, it is

necessary to dilute this solution before giving it to the rats, for the dose to be given is so small that the difficulty of giving it accurately would introduce a large error into the method. The dilution should be made by weight and should be of such a concentration that the amount to be given to each rat is contained in whatever weight or volume can be conveniently and accurately given. The dose of Standard should never be mixed with a rat's food. For dosing the writer uses the Burroughs Wellcome "Agla" micrometer syringe fitted with a blunt injection needle which can be held in a rat's mouth by one worker while another gives the turn of the screw required for delivering the dose of oil or solution. The volume of the dose has, of course, to be adjusted to the specific gravity of the oil. An average value, 0·925, for ordinary cod liver oils can be used, and an average value, 0·92, for olive oil can be used for the diluted Standard. If a series of tests is being carried out, a fresh dilution of the Standard should be made at no longer intervals than three weeks and kept in the cold store except when needed for dosing.

C. The general arrangement of the test for a determination of the vitamin D content of a substance in terms of the International Standard.

It has already been shown in the earlier part of this chapter that a simultaneous test on the Standard must be made whenever the vitamin D content of a substance is to be determined. It was mentioned that half of the rats of each litter used should be given doses of the Standard and the other half should be given doses of the test substance. This is necessary because the reserves of vitamin D in rats differ from litter to litter; and differences in reserves bring about differences in the severity of rickets developed and differences in the amount of healing after dosing with the same amount of vitamin D. As there is no method of determining the rats' reserves, the only precaution that can be taken is to divide each litter into two groups as nearly alike as possible and use one group for the Standard of reference and one for the test substance. This, of course, applies to both curative and prophylactic tests.

The rats allocated to the Standard may be divided into three groups and the groups given different doses of the Standard in the ratio 4 : 2 : 1, each rat in any one group receiving the same dose.

TABLE V

VARIATION MONTH BY MONTH IN THE AVERAGE HEALING OF RATS WHICH, HAVING BEEN GIVEN THE STEENBOCK 2965 RACHITOGENIC DIET FOR 18–24 DAYS, WERE THEN GIVEN A SINGLE DOSE OF VITAMIN D (INTERNATIONAL STANDARD) AND NO FURTHER SUPPLEMENT FOR 10 DAYS.

	1939					1940					1941					1942				
	No. of pairs of rats.	Dose I.U.	Average healing.	Dose I.U.	Average healing.	No. of pairs of rats.	Dose I.U.	Average healing.	Dose I.U	Average healing.	No. of pairs of rats.	Dose I.U.	Average healing.	Dose I.U.	Average healing.	No. of pairs of rats.	Dose I.U.	Average healing.	Dose I.U.	Aver. age healing.
January						39	5	1·64	10	2·35	18 26	2·5 4·25	1·33 2·02	5 8·5	2·14 2·35	20	3	1·21	9	2·08
February ..						37	5	1·81	10	2·54	9	4·25	1·67	8·5	2·22	31	3	1·00	9	1·81
March						43	5	1·35	10	2·22	54 61	3 3	1·25 1·36	6 6	1·79 1·85	30	3	0·89	9	1·67
April						25	5	1·50	10	2·06	47	3	0·94	9	2·09	13	3	2·31	9	3·23
May						54	5	1·38	10	1·92	62	3	1·05	9	2·07	19	3	3·61	9	4·45
June						26 28	5 7·5	1·13 1·41	10 15	1·57 1·91	70	3	1·94	9	2·96	16 23	3 2	4·44 1·01	9 6	5·09 1·76
July						20	5	1·98	10	2·65	42	3	2·15	9	3·45	15	2	0·40	6	1·72
August						34 8	7·5 7·5	2·84 2·63	15 15	3·03 2·94	20 66	2 2	2·31 2·29	6 6	3·65 3·35	18 16	3 3	1·11 1·19	9 9	1·93 2·25
September ..						46 67	5 2·5	2·25 2·06	10 5	2·60 2·49	71	2	3·31	6	3·96	9	5	1·33	15	2·11
October						10	2·5	1·85	5	2·25	39	2	2·06	6	2·94	48	5	1·43	15	2·06
November ..	38	5	1·63	10	2·47	10 15	4·25 2·5	1·85 1·02	8·5 5	2·65 1·90	39	2	0·74	6	1·90	54	5	1·29	15	2·07
December ..	64	5	1·48	10	2·19	55	2·5	1·46	5	2·33	14 12	2 3	0·66 1·31	6 9	1·21 1·46	63	5	1·43	15	2·14

Note :—Up to the line below March 1941, the doses were in the ratio of 1 : 2; after that the ratio was 1 : 3.

	1943					1944					1945				
	No. of pairs of rats.	Dose I.U.	Average healing.	Dose I.U.	Average healing.	No. of pairs of rats.	Dose I.U.	Average healing.	Dose I.U.	Average healing.	No. of pairs of rats.	Dose I.U.	Average healing.	Dose I.U.	Average healing.
January	23	5	1·48	15	2·26	18	8	2·53	24	2·72	30	8	1·31	24	2·40
February ..	61	5	1·81	15	2·49	32	8	1·75	24	2·45	19	8	2·34	24	3·05
						22	4	1·55	12	2·36					
March	80	5	3·59	15	4·20	50	8	1·98	24	2·65	42	6	1·43	18	2·10
April	32	5	3·72	15	3·75	22	8	3·32	24	3·52	85	6	1·58	18	2·11
	12	2	2·83	6	3·71										
May	76	2	2·70	6	3·61	52	2	1·33	6	2·42	67	6	1·51	18	2·19
June	58	2	2·75	6	3·67	12	2	1·08	6	2·58	86	6	2·30	18	2·94
						59	3	1·38	9	2·24					
July	35	2	4·43	6	4·76	108	3	1·22	9	2·01	73	6	2·55	18	3·55
											17	3	2·53	9	3·47
August	35	2	2·90	6	4·10	51	3	1·09	9	1·59	64	3	1·81	9	2·90
	13	1	2·38	3	3·0										
September ..	51	1	1·07	3	1·81	29	3	1·18	9	1·63					
	5	2	1·40	6	2·50	32	4	1·63	12	2·16					
October	69	2	1·83	6	2·73	46	4	1·65	12	2·34					
November ..	53	2	0·92	6	1·75	55	4	1·36	12	2·08					
	15	4	1·10	12	1·52										
December ..	17	4	1·19	12	1·71	23	4	0·92	12	2·02					
	33	8	2·11	24	2·64	37	8	1·53	24	2·09					

The rats allocated to the substance under examination are similarly divided into three groups for the testing of three different doses of the test substance in the ratio 4 : 2 : 1 (or, if no information is available of the probable potency of the test substance, in a wider range, say, 9 : 3 : 1). Each of these six groups should contain at least 6 animals. When the results from the different groups are averaged, it may be found that the results from the three groups given doses of the Standard in the ratio 4 : 2 : 1 correspond with the results from the three groups given doses of the test substance in the ratio 4 : 2 : 1 when good evidence of the

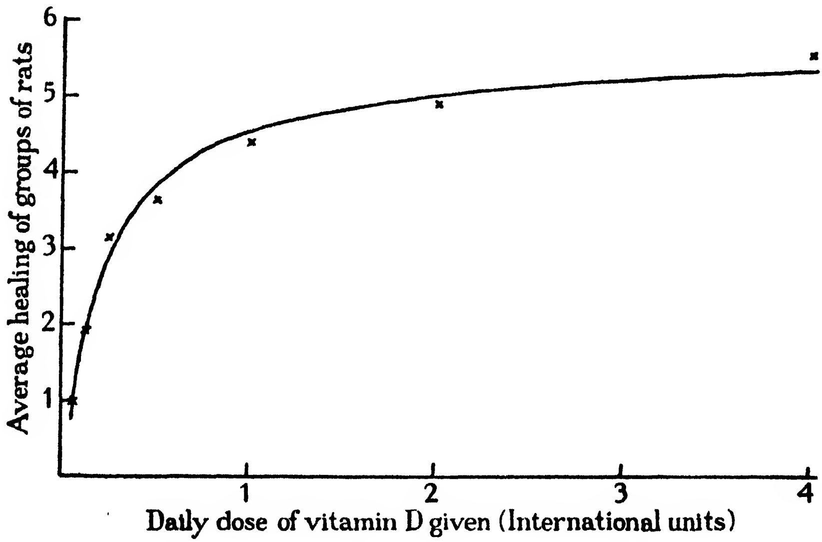


FIG. 22.—Curve of response relating healing of rats to dose of vitamin D given.

potency of the test substance would be obtained. It may happen that the results from the two higher doses of test substance are similar to the results from the two lower doses of Standard respectively, which would also give satisfactory evidence of the potency of the test substance. If, however, one dose only (say, the highest dose) of test substance corresponded to one dose only of the Standard, the lowest dose, then, in view of the variation in animal response, this evidence would not be very good. A further test should be made with doses which, judging from the first test, are more nearly equal to the doses of Standard, the

same doses of Standard being used again in the second test. This arrangement of the animals may be used whatever the criterion adopted for the measurement of the response to vitamin D and

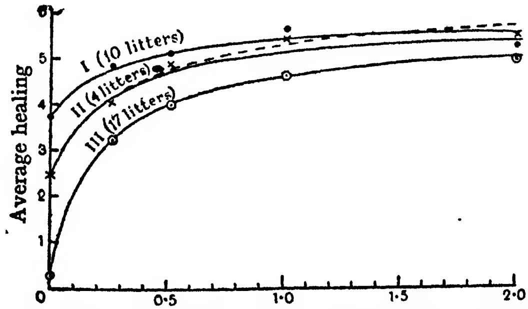


FIG. 23.—Different curves of response to vitamin D from rats having different degrees of rickets at the beginning of the test period, *i.e.* when the vitamin D was first given. (Dyer, 1931.)

whether the test is made as a curative or as a prophylactic one.

The determination of the shape of the curve of response.—Each

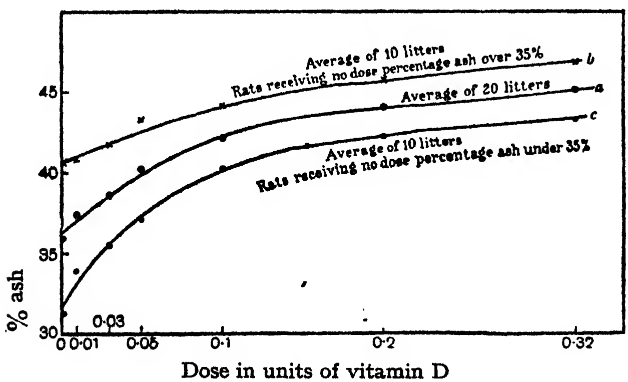


FIG. 24.—Curves of response relating the percentage ash in dry fat-extracted bone to dose of vitamin D given (Hume, Pickersgill and Gaffikin). Different curves of response are obtained according to the percentage ash in the rats which received no supplement to the rachitogenic diet.

worker should determine for himself the general shape of the curve of response obtainable with the criterion he chooses. Ten litters of rats should be used. After the preliminary period of

feeding on a rachitogenic diet the rats are placed in separate cages. One rat of each litter is given, say,

(*a*) no dose,
(*b*) 0·125 unit vitamin D daily,
(*c*) 0·25 unit vitamin D daily,
(*d*) 0·5 unit vitamin D daily,
(*e*) 1·0 unit vitamin D daily,
(*f*) 2·0 units vitamin D daily for the period of the test, or a single dose ten times the amount of the daily dose may be given and no further dose for 10 days. The results are averaged and plotted against the doses of vitamin D given to construct the curve of response (Figs. 22, 23 and 24). The curves may be regarded as logarithmic in shape.

2. The Preparation of Rats for a Determination of Vitamin D

A. Animals suitable for the test.

The colony of rats which is to supply animals for vitamin D work should be fed on a diet containing enough vitamin D for growth and reproduction but not enough to make the reserves of the young so large that they do not develop rickets when given a rachitogenic diet. The feeding of a colony of rats that produces young suitable for vitamin D work has been described in Chapter II. Young rats from this colony are generally suitable to begin their preparation for a vitamin D determination by the curative method at a weight of 50–60g., but this will vary somewhat according to the condition of the colony. For a prophylactic test, rats of a lower weight (about 40g.) are generally used.

B. Housing of the animals.

The laboratory in which vitamin D work is to be carried out should receive no direct sunlight. If it does, blinds should be provided and kept drawn while the sun is shining directly on the windows. Even "sky-shine" without direct sunlight has been found to cure experimental rickets.

All the rats of one litter may be kept together in one cage of dimensions about 12″ × 18″ × 8″ during the preparatory period. During the period of dosing (often called the "test period") each

rat should be kept in a separate cage of dimensions about 9″ × 12″ × 6″. Bedding may be of sawdust or shavings, and provided that this has not been exposed to strong sunlight or radiations from a carbon arc, wire screens are not necessary. Cages should be cleaned thoroughly once a week.

C. Rachitogenic diets.

Steenbock's rachitogenic diet, No. 2965 (Steenbock and Black, 1925), has been found satisfactory in a great many laboratories. It consists of :

Yellow maize, whole seed ground finely	76%
Wheat gluten	20%
Calcium carbonate	3%
Sodium chloride	1%

McCollum's diet, No. 3143 (McCollum, Simmonds, Shipley and Park, 1921), has also been found satisfactory in many laboratories. It consists of :

Whole wheat, ground	33%
Yellow maize, whole seed ground finely	33%
Gelatin	15%
Wheat gluten	15%
Calcium carbonate	3%
Sodium chloride	1%

It is absolutely essential that the constituents of these diets should be thoroughly mixed, for the development of rickets depends on the calcium-phosphorus ratio of the diet and it would be fatal to the success of the experiment if some rats ate more calcium carbonate than the others. It is therefore advisable to mix not more than 3 kilos. of diet at a time, mixing first the sodium chloride and calcium carbonate by rubbing together in a mortar, then adding the wheat gluten in two or three portions, then the yellow maize and other constituents, if any.

Water.—Some workers supply their rats with distilled water, and this may be necessary in some districts. The London tap-water (notably a "hard" water) has been found suitable. It should be given fresh each day in an inverted flask or bottle provided with a straight outlet tube which lets out drops of water only when a rat licks the lower end. The drinking water is thus kept clean.

3. Criteria for the Measurement of the Response of Rats to Doses of Vitamin D

The criterion usually employed for the determination of vitamin D is the calcification of bones of rats which have been rendered suitably rachitic by feeding them on a diet which has a high-calcium, low-phosphorus content and which is devoid of vitamin D. This reaction is almost specific for vitamin D. The only method other than the giving of vitamin D of bringing about improved calcification after a diet with a high-calcium, low-phosphorus content has been used for some time, is to reduce the Ca : P ratio to a value more nearly equal to 1 (Key and Morgan, 1932; Bruce and Callow, 1934). The presence of a high percentage of phosphorus in the substance under examination would therefore improve the calcification of the bones of the test animals independently of the action of vitamin D which might also be present. The vitamin D value of the substance would then appear to be higher than it really was and another determination of its potency in which a diet containing a different Ca : P ratio was used would fail to confirm the former result. Moreover, Bruce and Callow (1934, 2) and Coward and Kassner (1940) have shown that the effect of giving extra phosphorus with vitamin D (as might happen in testing a food for vitamin D) is greater than the sum of giving the same amounts of phosphorus and vitamin D separately. Hence no allowance for the presence of a known amount of phosphorus can be made in assessing the healing, and the determination of vitamin D should therefore be made on an ether extract of this food substance. [Adjustment of the Ca : P ratio by substituting a calculated amount of calcium phosphate for calcium carbonate would involve considerations of the nature of the phosphorus (whether organic or inorganic) in the food and also of its availability to the rat which it might not be possible to determine].

The growth-promoting (increase in weight) property of vitamin D was first demonstrated by Steenbock, Nelson and Black (1924). How far this property could be used for the determination of the vitamin was investigated by Coward, Key and Morgan (1932), who demonstrated a curvilinear (logarithmic) relationship between the dose of vitamin D given and the increase in weight of rats which were given different doses of vitamin D daily for 5 weeks after they had become steady in weight on a diet deficient

in this factor (Fig. 29). The individual variation of the response of rats to the same dose of vitamin D was found to be much greater than that of rats in the corresponding vitamin A test. There was therefore no temptation to substitute this test for the more specific one in which the promotion of calcification was the criterion used.

A. **The "line" test, a curative method.**

This test requires a preparatory period during which all the animals are given a rachitogenic diet *ad lib.* followed by a curative period during which doses of Standard and test substance are given in addition to the same rachitogenic diet.

(i) *Preparatory period.*—Two and a half to three and a half weeks will in general be sufficient to produce severe rickets in rats fed on Steenbock's diet, 2965, provided the diet of the stock colony is poor in vitamin D. This condition can be detected by the swollen condition of the wrists and knees. A halting gait may be detected.

The rats should be weighed once a week during this time. If any lose more than 2–3g. weight they should be discarded. Generally the rats grow, but at a rather slow rate.

The diet should be supplied in abundance. No record of the amount eaten need be kept.

(ii) *Curative period.*—The length of the curative period generally adopted is 10 days. The dose of Standard and of unknown may be given daily throughout the test period, or if the total amount for the 10 days test is not too much for a rat to take at once, it may be given as one dose at the beginning of the curative period (Coward and Key, 1934). In testing a substance such as butter, of which 0·5g. is a convenient daily dose, the whole 10 days' dose, 5·0g. would be too much to give to a rat on one day, and it should be given as daily doses. According to Coward and Key, the whole dose of Standard may still be given as one dose at the beginning of the curative period, but according to Bacharach (1936), the dose of Standard should be divided into daily doses also, for he found in his colony that a given amount of vitamin D was more effective when given in daily doses than when given as one dose.

A micrometer syringe should be used for giving small doses of cod liver oil or of the Standard, accurately. One worker should hold the rat firmly with its mouth open, and the end of the

syringe well within the rat's mouth, while another delivers the required dose from the syringe. The rats should be weighed twice during the test period. The result from any rat which has lost 3–4g. during the time should be regarded with suspicion for spontaneous healing may take place during loss of weight.

It is sometimes useful to know the amount of food eaten by each rat per day. Ten grams of food may be weighed into the food pot the first day and the part not eaten weighed the next day. This is made up to 10g. (or more if required) and the process repeated daily throughout the test. Loss of appetite may lead to loss of weight, which may cause spontaneous healing of rickets.

(iii) *Number of rats in a test.*—Workers have gradually come to the conclusion that the slopes of curves of response relating healing to dose of vitamin D given often differ significantly (see Tables VI*a* and VI*b*). Therefore each experiment or assay should have its own curve of response obtained as the average of two curves determined from (*a*) 2 doses of the Standard in the ratio 2 : 1 or 3 : 1 and (*b*) 2 doses of the substance under test in the same ratio. If only about 30 rats are available for a test, it is better to use 4 groups of 8 rats each than 3 groups of 10 each, two for the Standard and one for the substance under test; for then the whole of the 32 rats are giving information on the slope, instead of only 20 rats as in the latter arrangement. But in practice the constitution of the litters available has to be considered and it may be possible to select more litters of 3 or 6 animals than of 4 or 8. Male or female rats apparently respond equally to vitamin D, hence no equalising of numbers of the sexes is necessary, but "litter-mate" control must be strictly exercised.

Some workers prefer to use 3 doses of the Standard and 3 of the test substance, each series in the ratio 4 : 2 : 1. This does not influence the estimate of the *slope* of the curve from each substance (when equal numbers of rats occur in every group) for that is determined by the lowest and highest doses, but it does influence the *position* of the curve, for the middle dose may raise or lower it.

(iv) *Measurement of healing by the macroscopic examination of the cut bones.*—At the end of the 10 days' curative period the rats are killed by coal gas or ether. The forelegs are cut off about the middle of the radius and ulna, the skin drawn back over the paws and the distal ends of the radius and ulna removed.

TABLE VI*a*

Variation in the slope of the curve of response obtained month by month from pairs of rats given doses of x and 2x International Units, respectively, of Vitamin D.

	1939.		1940.		1941.	
	No. of pairs of rats.	Slope, *b*.	No. of pairs of rats.	Slope, *b*.	No. of pairs of rats.	Slope, *b*.
January			39	2·36	44	1·76
February			37	2·42	63	1·79
March			43	2·89	61	1·63
April			25	1·86		
May			54	1·79		
June			54	1·56		
July			54	1·83		
August			54	1·13		
September			67	1·43		
October			10	1·33		
November	38	2·79	25	2·82		
December	64	2·36	55	2·89		

TABLE VI*b*

Variation in the slope of the curve of response obtained, month by month, from pairs of rats given doses of x and 3x International Units, respectively, of Vitamin D.

	1941.		1942.		1943.		1944.		1945.	
	No. of pairs of rats.	Slope, *b*.	No. of pairs of rats.	Slope, *b*.	No. of pairs of rats.	Slope, *b*.	No. of pairs of rats.	Slope, *b*.	No. of pairs of rats.	Slope, *b*.
January			20	1·82	23	1·63	18	0·40	30	2·28
February			31	1·70	61	1·42	54	1·55	19	1·28
March			30	1·63	80	1·28	50	1·19	42	1·40
April	47	2·41	13	1·93	44	0·54	22	0·42	85	1·11
May	62	2·14	19	1·76	76	1·91	52	2·28	67	1·42
June	70	2·14	39	1·49	58	1·93	71	1·68	86	1·34
July	62	2·74	33	2·20	35	0·69	108	1·66	90	2·07
August	66	2·22	16	2·22	48	2·26	51	1·05	64	2·28
September	71	1·74	9	1·63	56	1·61	61	1·03		
October	39	1·84	48	1·32	69	1·89	46	1·45		
November	39	2·43	54	1·63	68	1·55	55	1·51		
December	26	0·78	63	1·49	50	1·11	60	1·61		

This variation is being investigated further.

A label bearing the rat's number written in pencil is tied to the two bones and the whole placed in 4% formaldehyde (10% formalin) for a few hours (or days if more convenient) to clear the tissues somewhat. The two bones are then separated by a cut with a sharp scalpel and each bone cut longitudinally in a plane parallel to the plane of the cut made to separate the two bones. The four halves are placed in 1·5% silver nitrate for a few minutes, the cut surface uppermost. They are then placed in distilled water and exposed to light. The phosphates of the bone are changed to silver phosphate which is converted into colloidal (black) silver on exposure to light Thus the metaphysis which consists of cartilage and is not stained is easily recognised and the blackened "line" in it (if any) can be measured and a value assigned to it. The healing of the rats given any one dose are averaged.

To average the healing of different bones, each one must be assessed by comparison with a scale of a series of bones showing graded amounts of healing to which numerical values have been given. A scale of this kind has been drawn up in the laboratories of the Pharmaceutical Society of Great Britain, and has been in use there for several years (Fig. 25). Six stages of healing are represented, each stage from 1 to 4·5 being produced by approximately double of the dose required to produce the stage below it on the scale. This was determined after the scale had been drawn up and a curve of response constructed by the method described in an earlier part of this chapter. The curve may be seen in Fig. 22. It is obviously logarithmic throughout the greater part of its length. Different workers do not always assess the amount of healing in a particular bone equally. It has happened that one worker in a laboratory almost consistently assesses a bone half a degree higher than another worker, but at the range at which this test is most accurate this affects the result very little. When the assessments vary by half a degree either way the averages from 10 rats generally agree so well that the results obtained by two workers do not differ by more than 5%.

The healing can, however, be measured mechanically, with possibly more accuracy. Morgan (1932) made camera lucida drawings of his "line test" bones with constant magnification and then measured the area of new calcification by means of a planimeter. When a less densely calcified part was distinguishable

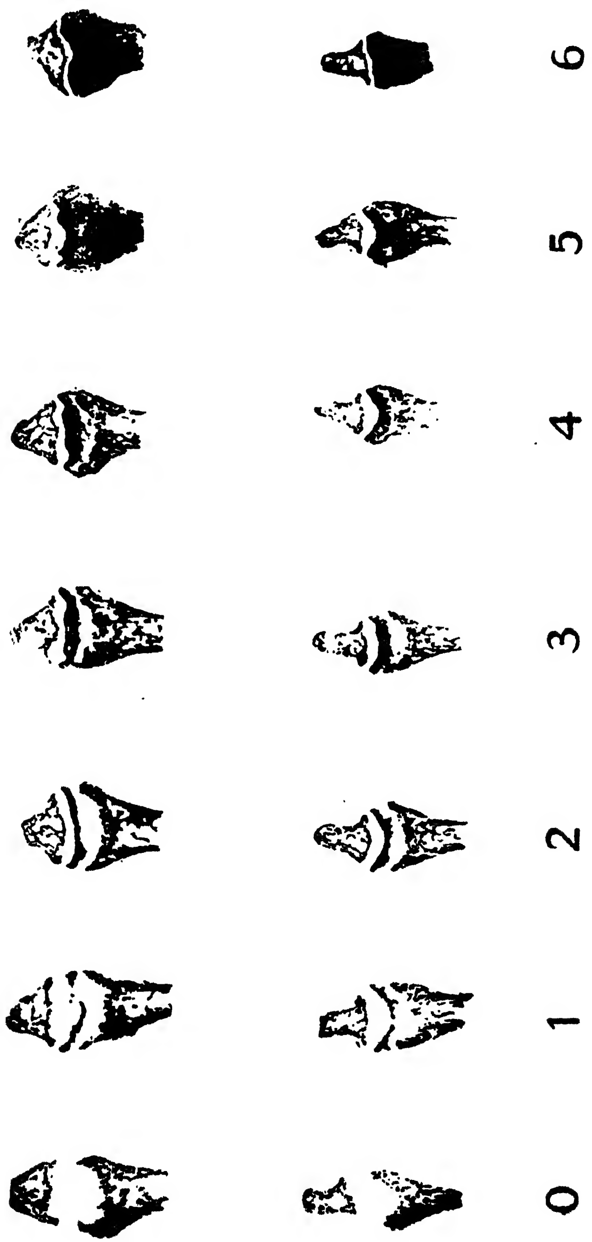


FIG. 25.—Scale of healing for measurements in the "line test."

from the very densely calcified part he assigned only half the value to the less dense part.

(v) *Working out the result.*—(*a*) If three groups only have been used for the test, two groups having been given doses of the Standard (say, 5·0 and 10·0 units respectively as one dose to each rat) and one group having been given a dose of cod liver oil (say, 50mg. to each rat) then the results from the rats in each group are averaged. Those from the two doses of the Standard are plotted against the logarithms of the doses and the points so obtained are joined by a straight line. This procedure assumes that the relation between effect and dose is logarithmic. The

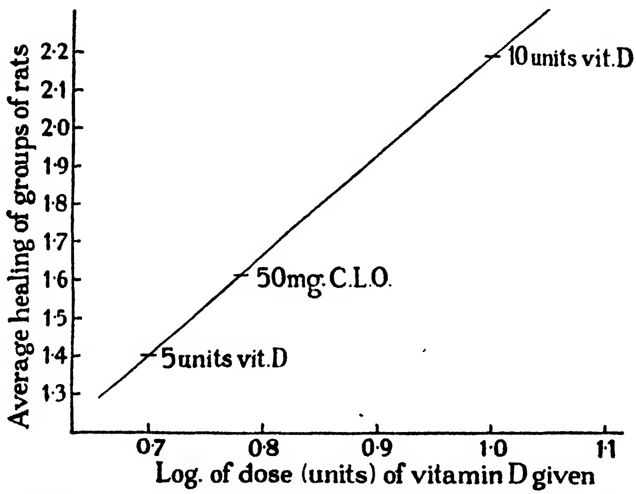


FIG. 26.—Method of working out a result when three groups of rats have been given two doses of vitamin D and one dose of cod liver oil respectively.

abscissa corresponding to the average result from the cod liver oil is found, and since the abscissa is the log. of the dose, the dose is found by determining the antilog. of the abscissa. This then is the number of units of vitamin D in the dose of cod liver oil given. The potency is stated as the number of International units per gram of oil.

Example.—Suppose two groups of rats, given 5 units and 10 units respectively of vitamin D per rat as one dose, showed average healing of 1·40 and 2·19 scale divisions. The results 1·40 and 2·19 are plotted as ordinates against the logs. of 5 and 10, *i.e.* against 0·6990 and 1·0, as abscissæ. A straight line is drawn between the two points so obtained (Fig. 26). Suppose

the average healing of the rats given 50mg. cod liver oil was 1·61. The abscissa corresponding to this on the curve is 0·78. The antilog. of 0·78 is 6·026. Therefore 50mg. cod liver oil contain 6·026 units of vitamin D and the cod liver oil contains 120 units of vitamin D per gram. It would be absurd to state the potency more accurately than this.

The straight line drawn between the points determined from the results from the doses of Standard may be prolonged a little way in either direction for the interpretation of results from the test substance lying outside the results from the Standard. But extrapolation is always risky and it should not be carried far.

The results may be calculated without actually drawing the curve of response.

A difference of 0·79 scale division (2·19—1·40) corresponded to a doubling of the dose of Standard, *i.e.* 0·79 scale division corresponded to a difference of 0·30103 in the logs. of the doses of vitamin D given. Hence a difference of 0·21 scale division (1·61—1·40) corresponded to a difference of $\frac{0{\cdot}30103 \times 0{\cdot}21}{0{\cdot}79} = 0{\cdot}0800$ in the logs. of the doses given. Therefore 50 mg. cod liver oil which produced an average healing of 1·61 is 1·202 (antilog. 0·0800) times as potent as 5 units of vitamin D which brought about an average healing of 1·40. Thus 50mg. cod liver oil contain 1·202×5 units=6 units of vitamin D, and the oil contains 120 International units of vitamin D per gram.

(*b*) When two doses of the Standard and two doses of the substance under test have been used, the calculation of the potency is made by the following method.

Find the average healing of the rats in each group.

Find the slope of each curve, *i.e.* the slope of the curve relating average healing from 2 doses of Standard to the logarithm of the doses given, and a similar curve for the test substance.

Find the average of these two slopes.

Place parallel curves with this average slope through the "mid-points" (*i.e.* the points with average values for x and y) of the two original curves.

Calculate the horizontal distance between the curve for the test substance and that for the Standard (beware of the sign).

Find the antilog. of the horizontal distance.

This is the ratio of the potencies of the substance under test and the Standard.

Example.

TABLE VII

THE DETERMINATION OF THE VITAMIN D CONTENT OF A SAMPLE OF COD LIVER OIL BY THE "LINE-TEST".

Dose.	Litter.	Rat.	Healing.	Average healing.
20mg. C.L.O.	16636	5055	1·5	
	16648	5110	1·0	
	16653	5137	0·5	
	16668	5213	0·25	
	16654	5143	1·0	
	16655	5149	1·5	1·25
	16665	5196	3·0	
	16675	5251	1·5	
	16687	5306	2·0	
	16677	5262	1·0	
	16679	5275	0·5	
60mg. C.L.O.	16636	5052	2·0	
	16648	5113	2·5	
	16653	5136	3·5	
	16668	5215	0·25	
	16654	5141	2·5	
	16655	5147	1·0	2·16
	16665	5198	3·5	
	16675	5249	3·0	
	16687	5304	2·5	
	16677	5358	3·0	
	16679	5271	0 trace	
4 units standard vitamin D	16636	5050	1·5	
	16648	5112	1·0	
	16653	5140	1·5	
	16668	5217	0·5	
	16654	5144	1·5	
	16655	5151	1·5	1·59
	16665	5195	3·5	
	16675	5252	2·5	
	16687	5308	1·0	
	16677	5263	1·5	
	16679	5270	1·5	
12 units standard vitamin D	16636	5053	2·0	
	16648	5111	2·0	
	16653	5138	1·5	
	16668	5214	0·25	
	16654	5142	1·5	
	16655	5148	2·0	2·34
	16665	5197	4·5	
	16675	5250	3·5	
	16687	5305	1·5	
	16677	5264	2·0	
	16679	5272	5·0	

Calculation.—(At length, to demonstrate the principles involved.)

	Dose.	Log. dose.	Average healing.
C.L.O.	20mg. 60mg.	1·3010 1·7781	1·25 2·16
		Diff.=0·4771	Diff.=0·91
International Standard	4mg. 12mg.	0·6021 1·0792	1·59 2·34
		Diff.=0·4771	Diff.=0·75

The average slope of the two curves is $\frac{0{\cdot}91+0{\cdot}75}{2\times0{\cdot}4771}=1{\cdot}74$

The "mid-point" of the curve for the C.L.O. is 1·5396, 1·705.
The "mid-point" of the curve for the Standard is 0·8407, 1·965.
The curve with average slope, 1·74, through the "mid-point" of the curve for the C.L.O. is

$$\frac{y-1{\cdot}705}{x-1{\cdot}5396}=1{\cdot}74$$

therefore $$y=1{\cdot}74x-0{\cdot}97$$

The curve with average slope, 1·74, through the "mid-point" of the curve for the Standard is

$$\frac{y-1{\cdot}965}{x-0{\cdot}8407}=1{\cdot}74$$

therefore $$y=1{\cdot}74x+0{\cdot}50$$

The *vertical* distance between these two lines is $-0{\cdot}97\ -0{\cdot}50$
$=-1{\cdot}47$

Then since $\frac{y}{x}=$ the slope $=1{\cdot}74$

$$\frac{-1{\cdot}47}{x}=1{\cdot}74$$

therefore $$x=-\frac{1{\cdot}47}{1{\cdot}74}=-0{\cdot}845$$

i.e. the horizontal distance between the curves is $-0{\cdot}845$
which $=\bar{1}{\cdot}155$
which $=\log.\ 0{\cdot}143$

Therefore the cod liver oil has 0·143 of the potency of the International Standard, and since the International Standard

contains 1,000 units per gram, this sample of cod liver oil contains 140 International units per gram. (It is not worth while to give a more exact figure than this.)

The calculation can be simplified somewhat since there are 2 doses each of the Standard and cod liver oil, and the two doses are in the ratio 3 : 1.

Suppose, for the sake of the calculation, that the doses had been 1 and 3mg. of both the C.L.O. and the Standard.

Then the difference between the logs. of the doses would still have been 0·477, and the "mid-point" of the C.L.O. curve would have been 0·239, 1·705, and the "mid-point" of the Standard curve would have been 0·239, 1·965.

The curves with average slope, 1·74, would have passed through the point, $x=0{\cdot}239$, in both curves and the vertical distance between these parallel curves would have been $1{\cdot}705-1{\cdot}965 = -0{\cdot}260$ at this value of x and at all other values of x.

Then $$\frac{y}{x}=1{\cdot}74$$

therefore $$\frac{-0{\cdot}260}{x}=1{\cdot}74 \text{ and } x=\frac{-0{\cdot}260}{1{\cdot}74}$$
$$=-0{\cdot}1494$$
$$=\bar{1}{\cdot}851$$
$$=\log. 0{\cdot}71$$

Therefore the cod liver oil would have been said to have 0·71 of the potency of the International Standard ; but the doses of C.L.O. were 5 times the doses of the Standard. Therefore the C.L.O. has $\frac{1}{5}$ of 0·71 of 1,000 International units, *i.e.* 140 International units per gram.

Since the number of animals is necessarily the same in all groups, owing to "litter-mate" control, it has no influence on either of these forms of calculation.

B. The "X-ray" method, generally used as a curative test.

(i) *Preparatory and curative periods.*—Bourdillon, Bruce, Fischmann and Webster (1931) worked out the details of this method which they used extensively in their work on the preparation of calciferol from irradiated ergosterol. They found that a preparatory period of 14 days' feeding on Steenbock's rachitogenic diet, No. 2965, was suitable for rats from their colony. Their

curative period was 14 days also. The other details of the procedure for carrying out the determination were similar to those described for the line test.

(ii) *Measurement of healing.*—The bones that were examined by X-ray photography were the proximal ends of the tibiæ. Each rat was photographed twice, once under anæsthesia before dosing and once when killed after 14 days of dosing. The scale of healing used for assessing the healing of the experimental rats was divided into 12 divisions. The procedure for photographing the rats' bones is here quoted at length from the Report on the Quantitative Estimation of Vitamin D by Radiography, by Bourdillon, Bruce, Fischmann and Webster (1931).

"*Apparatus.*—The X-ray plant consisted of a dental-type, hot kathode tube supplied with 4 amps-filament current, and about 12ma. high tension current at about 35,000 volts, from transformers with suitable regulating resistances. The voltage was kept as constant as practicable, and an exposure of exactly 5 seconds was ensured by an automatic shutter which was kindly constructed for this purpose by Dr. E. Schuster. The distance from the focal spot on the kathode to the plate was 16".

"The rats were held in a carrier which retained one foot by a light spring clip, in such a position that the knee-joint was kept extended. This carrier allowed for the photography of 10 legs on one 10" × 4" plate. All plates were developed for a constant time, and at a constant temperature, as uniform exposure and development is of importance in order to facilitate accurate evaluation of calcium deposits. Our photographs appear nearly uniform in contrast (except the earlier series taken with a 'gas' X-ray tube), but sometimes vary perceptibly in density, owing, we believe, to slight variations in the temperature of the developer. However, such variation did not occur between the photographs of any one plate, and as only one plate was used for each litter it could not appreciably influence results.

"*Anæsthesia.*—Before the first radiograph the animals were anæsthetised by being placed on a shelf in a 2-litre glass desiccator containing ethyl chloride vapour. The ethyl chloride was carefully measured before vaporisation, the amount used being 1·5c.c. for the first animal in a series, and 0·4c.c. or less for each subsequent animal.

"The depth of anæsthesia was judged by observing the rate of loss of consciousness, and the changes in respiration. The

average time of exposure to the vapour was 15–30 seconds. The animals appeared to recover completely almost immediately after the radiography.

"It was found that one operator could anæsthetise and radiograph 35–40 live rats per hour, and could radiograph dead rats at a much faster rate."

The X-ray photograph gives a picture of the healing bone very similar to that given by the line test, except, of course, that the line test shows only the section of the layer of healing and is, therefore, sharper in outline than the X-ray picture which shows the whole thickness of the layer of healing. Each method obviously needs experience in reading the healing.

Assessment of healing from the X-ray photographs is made by comparison with a scale of bones showing graded stages of healing. Bourdillon's scale, reproduced in Fig. 27, consists of 12 stages. He found that doubling the dose of vitamin D resulted in additional two-scale divisions of healing. This is comparable with the finding of Coward and Key (1933), since Bourdillon has twelve divisions in his scale of healing and Coward has six. It indicates also that the rats of the two colonies must have been reduced to rachitic states of similar severity, although the treatment of the two was different.

(iii) *Working out the result.*—Bourdillon's method of working out results was similar to one of those originally used for working out the results obtained by the line test. If one member of a pair of rats from the same litter had been given a dose of cod liver oil and the other member of the pair a dose of the Standard, the difference in healing (as scale divisions) between the 2 rats was determined. Then, since a difference of two scale divisions corresponded generally to a doubling of the dose of vitamin D, the difference in healing was proportional to the difference between the logs. of the doses of vitamin D given. A simple proportion sum and converting the answer into its antilog. gave the ration of the vitamin D content of the doses given to the 2 rats. Another proportion sum and the averaging of results from, say, 10 such pairs of animals, gave the potency of the preparation.

It would probably be agreed nowadays, however, that a determination made by the "X-ray" method should be arranged as described for the "line-test" method, *i.e.* use 4 groups of animals two of which should be given doses of the Standard in the ratio

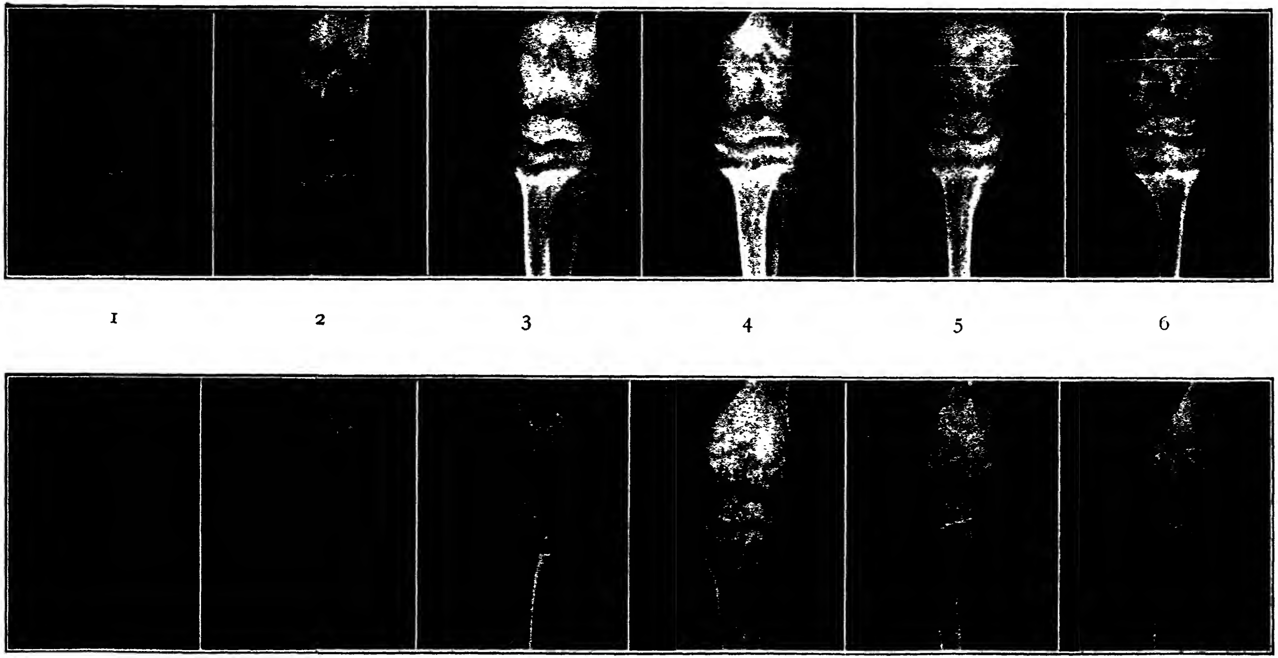


FIG. 27.—Scale of healing in rachitic rats enlarged nearly 2 diameters.

[To face page 130.

2 : 1 or 3 : 1 and two given doses of the substance under test in the same ratio. The calculation would then be made as described for the "line-test" on p. 125.

C. The "bone-ash" method, generally used as a prophylactic test.

(i) *The test period.*—In this method of determination of vitamin D, the doses of test substance and of Standard are given from the beginning of the experiment, doses being chosen of about one-fifth the amount of the doses suitable for a curative test. Doses may be given once or twice a week instead of daily. Each rat should be kept in a separate cage throughout the experiment. Either of the rachitogenic diets already described is suitable for this type of test.

(ii) *Number of rats in a test.*—About six litters of 7 rats each are divided into seven groups, 1 rat of each litter in each group. The rats of one group are given no dose of test substance or of Standard, the rats of the other groups being given doses of test substance in the ratio 4 : 2 : 1 or of Standard in a similar ratio, every rat of any one group being given the same dose. This is continued for 4 or 5 or 6 weeks, the longer the experiment the greater being the "spread" of the results obtained at the end.

If there is no information available as to the possible potency of the test substance a preliminary test must be made with doses chosen over a much wider range than the one proposed, say, in the ratio 9 : 3 : 1 or even wider still, the doses of the Standard being two (ratio 3 : 1 or 2 : 1) known to be suitable to this type of experiment.

(iii) *Measurement of calcification.*—The percentage of ash in the dry fat-extracted dry bone is required in this method of determination. The femora (or the humeri) of the rats are removed and freed as completely as possible from adhering tissue. Rubbing with cheese cloth is helpful in this. Each bone is broken in two, tied up in a piece of muslin and extracted for at least 6 hours with alcohol in a Soxhlet extractor. The time in the Soxhlet can be shortened if desired by previous boiling in a large volume of alcohol. The bones are then dried to constant weight. Each bone is ashed, by heating in a crucible first over a Bunsen burner, then in a muffle furnace, to constant weight, and the ash content thus determined. The result is expressed as the percentage of ash in the dry, extracted bone. The results from the

animals in each group are averaged and the averages compared. If the doses have been suitably chosen one experiment with 42 rats will give a very fair estimate of the potency of the test substance; if not, a second experiment will be necessary with doses of Standard and test substance respectively of more nearly equal potency.

Example.—A particular sample of cod liver oil was examined by this method. The results are collected in Table VIII. The potency of the oil may be determined from the following considerations:

(*a*) 0·8mg. cod liver oil contains more than 0·1 International unit of vitamin D; therefore 1mg. contains more than 0·125 unit.

(*b*) 0·4mg. cod liver oil contains more than 0·05 International unit of vitamin D, but less than 0·1 unit; therefore 1mg. cod liver oil contains more than 0·125 and less than 0·25 unit.

(*c*) 0·2mg. cod liver oil contains more than 0·025 International unit of vitamin D, but less than 0·05: therefore 1mg. cod liver oil contains more than 0·125 and less than 0·25 unit.

(*d*) 0·2mg. cod liver oil contains more nearly 0·05 unit than 0·025 unit; therefore 1mg. cod liver oil contains more nearly 0·25 unit than 0·125 unit.

Therefore the vitamin D potency of the oil may be estimated at about 200 International units per gram.

A more accurate way of dealing with results such as these is to plot each series of results against the logarithms, to the base 10, of the doses given (mg. cod liver oil or mg. International Standard) on the same graph, draw the best straight line through the two series (either by eye or mathematically as described on p. 5) and then, if the two lines are nearly parallel, as they are in this experiment, find the abscissæ of each curve corresponding to one particular percentage of ash and equate the doses. In Fig. 28 curve A is the curve of response to doses of the Standard in mg., curve B is the curve of response to doses of the cod liver oil in mg.

30% ash corresponds to abscissa $\bar{2}{\cdot}625$ on the curve for the Standard,

30% ash corresponds to abscissa $\bar{1}{\cdot}315$ on the curve for this cod liver oil.

Then the antilog. of $\bar{2}{\cdot}625$ as a dose of the Standard in mg. (or unit) corresponds to the antilog. of $\bar{1}{\cdot}315$ as a dose of cod liver oil in mg., *i.e.* 0·04217 unit is equivalent to 0·20654mg. cod liver

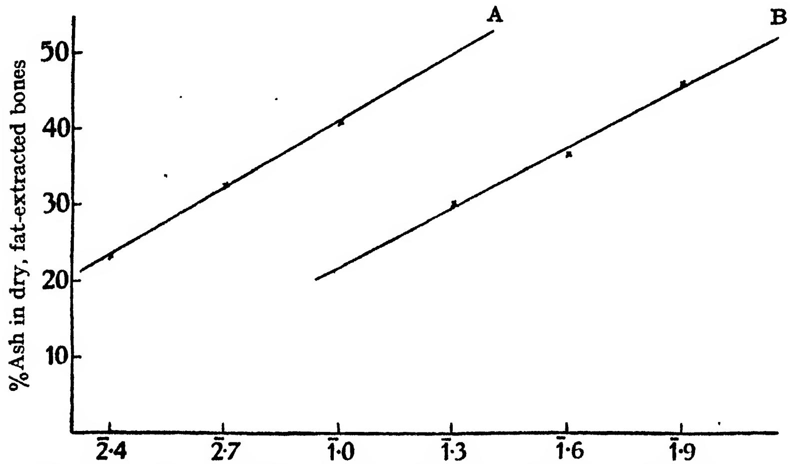
TABLE VIII

THE DETERMINATION OF THE VITAMIN D CONTENT OF A SAMPLE OF COD LIVER OIL BY THE "ASH CONTENT OF THE BONES" METHOD

Cod liver oil.						International Standard.					
Daily dose.	Rat.	Weight of dry extracted bone.	Weight of ash.	Percentage ash in dry extracted bone.	Average percentage ash.	Daily dose.	Rat.	Weight of dry extracted bone.	Weight of ash.	Percentage ash in dry extracted bone.	Average percentage ash.
0·8mg.	♀ 185	0·1172	0·0504	43·00	45·86	0·1 unit	♀ 186	0·1211	0·0430	35·51	40·79
	♀ 238	0·1249	0·0575	46·04			♀ 240	0·1057	0·0437	41·34	
	♂ 294	0·1606	0·0759	47·26			♂ 295	0·1345	0·0563	41·86	
	♂ 347	0·1427	0·0660	46·25			♂ 348	0·1227	0·0525	42·79	
	♀ 256	0·1230	0·0600	48·75			♀ 258	0·1119	0·0472	42·18	
	♂ 368	0·1369	0·0600	43·83			♂ 371	0·1244	0·0511	41·08	
0·4mg.	♀ 182	0·0976	0·0344	35·25	36·52	0·05 unit	♀ 183	0·0830	0·0312	37·59	32·52
	♂ 235	0·1069	0·0389	36·39			♀ 239	0·0945	0·0288	30·48	
	♂ 296	0·1317	0·0476	36·14			♂ 297	0·1228	0·0404	32·90	
	♀ 351	0·1098	0·0416	37·89			♂ 346	0·0848	0·0282	32·25	
	♀ 257	0·0815	0·0291	35·71			♀ 259	0·0914	0·0270	29·54	
	♂ 369	0·1089	0·0411	37·74			♂ 370	0·0878	0·0284	32·36	
0·2mg.	♂ 181	0·1000	0·0249	24·90	30·16	0·025 unit	♀ 184	0·0780	0·0161	20·64	23·38
	♀ 237	0·0909	0·0293	32·23			♂ 236	0·0883	0·0222	25·14	
	♀ 298	0·0961	0·0324	33·71			♀ 299	0·0992	0·0238	23·99	
	♀ 350	0·0894	0·0281	31·43			♀ 349	0·0829	0·0191	23·04	
	♂ 254	0·1080	0·0265	24·54			♂ 255	0·0842	0·0208	24·70	
	♀ 373	0·0922	0·0315	34·16			♀ 372	0·0804	0·0183	22·76	

oil. Therefore the potency of the cod liver oil is 204 International units of vitamin D per gram. It would be unwise to call this anything but 200 units per gram.

If the curves are more divergent than these, the data should be treated as described for results from the line-test. (See also Chapter XII.)



Logarithm of the dose of Cod Liver Oil or of the International Standard given

FIG. 28.—To determine the potency of a sample of cod liver oil by the "ash-content of the bone" method. Three groups of rats have been given doses of 0·2, 0·4, 0·8mg., respectively, of cod liver oil, and three other groups doses of 0·025, 0·05 and 0·1mg., respectively, of the International Standard for vitamin D.

D. The "increase in weight" method.

One attempt has been made to use the increase in weight of the whole animal as the criterion for the determination of vitamin D (Coward, Key and Morgan, 1932). The basal diet used in the investigation was made as nearly like the one used in the determination of vitamin A as possible, the difference being an absence of vitamin D in the vitamin D test instead of an absence of vitamin A as in the vitamin A test. In particular the same salt mixture was used in both tests; thus there was no disturbing high-calcium, low-phosphorus content for the vitamin D to rectify. The diet consisted of:

Caseinogen, light white (B.D.H.)	15%
Dextrinised rice starch	73%
Dried brewer's yeast	8%
Salt mixture (Steenbock's 40)	4%

In addition, each rat was given a dose of carotene equivalent to 0·04mg. in 0·02g. olive oil daily, three times a week throughout the whole of the experiment. The composition of the salt mixture was important in this experiment. It consisted of :

Sodium chloride (NaCl)	23·36 parts
Magnesium sulphate ($MgSO_4.7H_2O$)	24·6 ,,
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	35·8 ,,
Dipotassium hydrogen phosphate (K_2HPO_4) ..	69·6 ,,
Calcium phosphate ($CaHPO_4.4H_2O$)	68·8 ,,
Calcium lactate.$5H_2O$	15·4 ,,
Iron citrate.$6H_2O$	5·98 ,,
Potassium iodide (KI)	0·16 ,,

The experiment was carried out like the corresponding experiment with vitamin A. Rats were given this diet until they ceased to put on weight. During this time the members of any one litter were kept together in one cage. They were weighed twice a week, and as each one became steady in weight it was transferred to a separate cage for the rest of the experiment.

(i) *Behaviour of the rats while they were given the basal diet only.*—The increase in weight of the rats during the time that they were given the basal diet only varied greatly. Some increased only a few grams in weight, others increased as much as 60–70g., and a few had to be discarded for they attained too high a weight. Those that grew did so at a slow rate, much slower than rats given a vitamin A-free diet, and when they ceased to gain in weight, and were given no dose of vitamin D, they remained stationary for many weeks before dying, and sometimes died without much loss in weight. This again was very different from the behaviour of animals given a vitamin A-free diet, which lose weight rapidly after once becoming stationary in weight. This suggests that the need of the rat for vitamin D is less than its need for vitamin A.

(ii) *Response to doses of vitamin D.*—Daily doses of 0·01, 0·05, 0·1, 0·2, 0·25, 0·4, 0·75, 0·8 unit in olive oil were tested on groups of rats which had become steady in weight on the basal diet. Two of these doses (0·25 and 0·75) are unnecessary. For a fuller account of the experiment see Chapter VIII. The groups consisted of much smaller numbers of rats than had been used in constructing the curve of response to vitamin A in the same laboratory, but the average responses showed clearly that there was a graded response to graded doses of vitamin D. The

curvilinear relationship was evident even when the results were separated into those from male and female rats respectively, *i.e.* into groups consisting of much smaller numbers of animals. Both curves of response were logarithmic (Fig. 29).

(iii) *Test of the curve of response.*—The validity of this curve was tested by using this method to examine the potency of a sample of irradiated ergosterol whose potency was determined by the line test as 2,000,000 units per gram.

The test was carried out at the same time that the curve was being constructed. Doses of 0·00001 and 0·0005mg. respectively were tested, the high dose being begun before the very high potency of the solution was known from the line test. Thus the

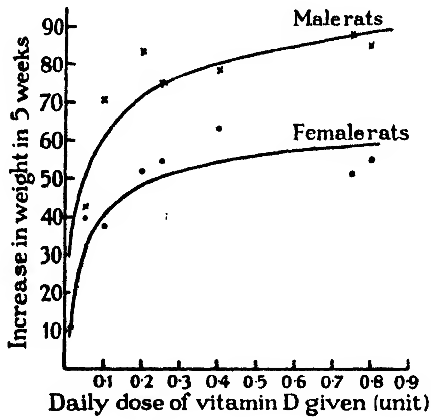


FIG. 29.—Curves of response of male and female rats relating increase in weight in 5 weeks to the dose of vitamin D given.

range ratio of the two doses was 50 : 1. The doses of the Standard which would have brought about the same increases in weight as the doses (0·00001 and 0·0005mg. solution respectively) were found from the curves of response and the potency of the solution thus determined. The figure actually found was 2,068,500 units per gram, which, by its confirmation of the potency determined by other workers using the line test, indicated that the increase in weight of the rats had determined the same substance that had been determined by the other method.

An investigation of the accuracy of this method proved to be disappointing, however. It was much less than that of the determination of vitamin A by a similar method.

E. Comparison of the four methods.

The best criterion to use for determining the vitamin D content of a substance is the ash content of the bones. This measures the whole calcification of a bone or bones which may be considered representative of the whole skeleton. It is the most accurate of the methods in general use (see Chapter XII). It has, however, the disadvantage of being very laborious and time-consuming. It requires at least 4, and preferably 5 or 6, weeks of feeding the animals with special doses daily or twice weekly, and the determination of the ash content of the bones after this adds greatly to the labour involved.

The "line test" and "X-ray" method involve about the same amount of work both in feeding the animals and in examining the bones afterwards. Both are more easily carried out than the "ash content of the bones" method for they involve shorter periods of special feeding and shorter treatment of the bones. They are, however, less accurate and subject to the personal error involved in assigning a value to each bone from a comparison with a series of bones showing a scale of healing. Both can be recommended for routine testing, the "X-ray method" having one disadvantage over the "line test" in the cost of the apparatus needed for taking the X-ray photographs.

The "increase in weight" method has little to recommend it. The stage at which the rat is ready for doses is ill-defined, the period of feeding is long (at least 3 weeks) and the accuracy of the test is low. The criterion is not even specific for vitamin D. Its one advantage lies in the fact that the measurement of increase in weight is independent of any personal judgment.

4. The Difference in Results obtained by using (a) Rats and (b) Chickens, in the Determination of Vitamin D

Massengale and Nussmeier (1930) first showed that cod liver oil was much more effective in promoting calcification of bones of chickens than was irradiated ergosterol when the two were given in doses equivalent in potency according to previous determinations on rats. This has since been confirmed by other workers. It has led to the conclusion that the vitamin D of cod liver oil is not the same substance as the vitamin D of irradiated ergosterol and that the chicken cannot make use of the vitamin

D of irradiated ergosterol to anything like the same extent as it can make use of the "natural" vitamin D of cod liver oil. This means that if a sample of cod liver oil were compared with the International Standard by testing on chickens it would be found to have a very much higher vitamin D value than if the comparison were made on rats. Bills, Massengale and Imboden (1934) have even suggested that there are at least two "natural" vitamins D and that they occur in different proportions in different fish liver oils.

This raised the question, how does the human subject react to the two (or more) forms of vitamin D? Hess thought that human babies made slightly less good use of the vitamin D of irradiated ergosterol than of the natural vitamin D, but an extensive investigation made by Eliot, Nelson, Barnes, Browne and Jenss (1936) on 600 children showed that they used the two forms of vitamin D equally well; that is, in this respect the human subject behaves like the rat and not like the chicken. Waddell (1934) produced the chick antirachitic factor by irradiating cholesterol under different conditions and ultimately vitamin D was prepared in crystalline form from irradiated 7-dehydrocholesterol by Windaus, Lettrè and Schenck (1935), and shown to be identical with the "natural" vitamin D of tunny liver oil by Brockmann (1936), Brockmann and Busse (1937, 1938) and with that of halibut liver oil by Brockmann (1937). Various workers found that the vitamin D activity of D_3 was equal to that of D_2 and eventually a collaborative test to compare the activity of pure D_2 and D_3 was organised by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute. A pure sample of calciferol was generously supplied by the Glaxo Laboratories and a pure sample of vitamin D_3 by Professor Hörlein of the I.G. Farbenindustrie A.G. to Sir Henry Dale. Workers in nine different laboratories made the comparison on rats and the results were so nearly alike that statistical analysis was unnecessary. Vitamin D_3 was declared to contain 40,000 International units of vitamin D per mg. Moreover, a comparison of these two preparations on the healing of rickets in children carried out by Morris and Stephenson (1939) in Glasgow, on cases of osteomalacia and late rickets by D. C. Wilson (1940) in India and a case of parathyroid tetany by H. P. Himsworth and M. Maizels (1940) in London, failed to shew any difference in antirachitic potency and indeed provided a certain

amount of positive evidence that the two forms were equally potent for human beings.

Therefore a preparation of calciferol which is intended for clinical work on children, may be assayed by comparison with the International Standard by tests on rats.

It is obvious that it is useless to add calciferol to cod liver oil which is intended for chickens. The potency would be scarcely any greater than that of the unfortified oil. It follows that when a sample of cod liver oil has been assayed by experiment on rats (the only method recognised at present by the Permanent Commission on Biological Standardisation of the League of Nations), the poultry dealer must demand a guarantee from the vendor of the cod liver oil that it is, in fact, a natural cod liver oil without any added calciferol. Otherwise the vitamin D potency of the oil must be determined by experiment on chicks in comparison with some standard preparation of the "natural" vitamin D. The British Standards Institution first adopted a particular sample of pure cod liver oil for this purpose as various workers showed that the cod is, so far, the only known fish whose liver oil contains only one form of vitamin D. When it was shown that this form was the one formed by the irradiation of 7-dehydrocholesterol, vitamin D_3, a sample of that was obtained to serve as a standard of reference for determining, by tests on chicks, the vitamin D potency of liver oils destined for poultry feeding.

The Provisional Standard Preparation of vitamin D_3.—The Provisional Standard Preparation of vitamin D_3 is a solution of pure crystalline vitamin D_3 in pure vitamin D-free olive oil. 1mg. of the solution contains 0·000025mg. of the crystalline vitamin. The preparation is kept under the control of the British Standards Institution.

The B.S.I. unit of antirachitic vitamin D_3 activity.—The B.S.I. unit of antirachitic vitamin D_3 activity is defined as the specific activity of 1mg. of the Provisional Standard Preparation. It must be realised, however, that there is no International Standard for vitamin D_3 and hence there can be no claim for a vitamin D_3 potency in terms of an International unit. The only standard of reference for vitamin D_3 at present available is that held by the British Standards Institution, and comparison with it can give a potency in B.S.I. units only. Of course, any worker who is prepared to rely on the purity of another sample of vitamin

D_3 may compare an oil with it and state the oil's potency as *x*mg. or μg. of vitamin D_3 per gram; but he runs the risk of other people not having as strong faith in the purity of his standard as he himself has.

The British Standards Institution has published a booklet (1940) on the method it suggests for the determination, by the use of chicks, of the vitamin D_3 content of an oil. It gives full details of the breed of chicks suitable for the test, diet, housing conditions, etc. The arrangement of the test is the same as that for a test with the use of rats, except that "litter-mate" divisions of the animals are not necessary, nor need there be the same number of birds in each group. But it is desirable to use 2, 3 or 4 groups of birds on the Standard and 2, 3 or 4 groups on the oil, the doses of both the Standard and oil being in the ratio 2·25 : 1·5 : 1, *i.e.* they are in geometric progression. The Standard and oil are each mixed with the diet in chick tests. The criteria suggested are (*a*) percentage ash in the tibia or (*b*) X-ray photography of the tibia and measurement of the tarsometatarsal distance. The curative type of experiment and the "line test" as used on rats cannot be used on chickens, for healing in chickens does not shew itself as a line of calcification across the metaphysis in the cut bone; it is diaphyseal and immediately contiguous with the trabeculoe that remain after rickets is produced. Results are calculated as described above for the "line test" and "bone ash" methods using rats. The B.S.I. booklet also contains a description (and example worked out) of the method of determining the accuracy (true fiducial limits) of a test.

Lachat (1935) has collected a very large amount of information (by means of a questionnaire) concerning the details of chicken tests as carried out in twenty-three different institutions. He has summarised this in a report which will prove of immense value to workers who are already engaged in this field and to others who contemplate such work.

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CHAPTER VII

THE DETERMINATION OF VITAMIN E

1. The International Standard of Reference and the Unit of Vitamin E Activity.
    - A. The need for a simultaneous test on the Standard of reference whenever a determination of vitamin E is made.
    - B. The dilution of the Standard of reference for dosing.
    - C. The general arrangement of the test for a determination of the vitamin E potency of a substance in terms of the International Standard.
2. The Preparation of Rats for a Determination of Vitamin E.
    - A. Animals suitable for the test.
    - B. Housing of the animals.
    - C. Diets.
3. Criteria for the Measurement of the Response of Rats to doses of vitamin E.
4. References.

VITAMIN E prevents resorption of the foetus in the rat. A partial deficiency of vitamin E in the diet of the doe causes paralysis in the young during lactation (Barrie). It is claimed to prevent abortion in cattle and miscarriage in the human being, but it is difficult to obtain convincing evidence of the latter. Shortly before the war a collaborative experiment in which workers in 13 different laboratories in Europe and the U.S.A. took part, was carried out to see whether *dl*-α-tocopheryl acetate might be a suitable substance to serve as an International Standard of Reference for determinations of vitamin E. Results from 4 of the laboratories were unsatisfactory (the slopes of the curves of response were not steep enough to be considered significant), but those from the other nine were used to gain an idea of the relation between dose and response. The criterion used was an "all or none" reaction, the production or failure of production of a litter by a positively mated female rat, and the number of positive responses in a group expressed as a percentage of the number of possible responses. The result of the whole co-operative test was satisfactory, but the war had started and it was impossible to

hold the Third International Conference on the Standardization of Vitamins which had been planned. The British members of the Conference, however, in consultation with Sir Henry Dale, a member of the Permanent Commission of the League of Nations, consented to accept the responsibility of adopting the proposed Standard of synthetic racemic α-tocopheryl acetate for international use, and authorised the National Institute for Medical Research, Hampstead, to proceed with its distribution. This emergency action was taken with the knowledge and approval of the League of Nations Health Organisation.

1. The International Standard of Reference

The International Standard of Reference for vitamin E is a preparation of synthetic racemic α-tocopheryl acetate whose properties are:

(i) Appearance: a pale golden yellow, clear, viscous oil; almost without odour.

(ii) Specific gravity: $d_4^{20°}$ 0·9545–0·9665 (correction for 1° temperature variation, 0·0007).

(iii) Refractive index: $n_D^{20°}$ 1·4958–1·4972 (correction for 1° temperature variation, 0·0004).

(iv) Absorption spectrum: in absolute alcohol shows a broad band λ max. 2,855Å $E^{1\%}_{1cm.}$ 42·5±1.

(v) Contains not more that 2% of free tocopherol and 96–100% (±2%) of tocopheryl acetate. 1mg. of α-tocopheryl acetate ($C_{31}H_{52}O_3$) is equivalent to 0·911mg. of free α-tocopherol ($C_{29}H_{50}O_2$).

The International Unit of Vitamin E is the vitamin E activity of 1·0mg. of the Standard Preparation. This is about the average value (0·5 to 1·5mg.) for the total median fertility dose which prevents resorption gestation in rats deprived of vitamin E when the substance is administered orally: when it is administered parenterally its activity is less.

The International Standard preparation is issued in the form of a standard solution in olive oil, the strength of the solution being such that 1 gram contains 10 International units or 10mg.

synthetic racemic α-tocopheryl acetate. The oil used for the preparation of the standard solution had the following properties :

(i) Specific gravity : 0·915–0·918 (15·5°/15·5°).
(ii) Refractive index (at 40° C.) : 1·4605–1·4635.
(iii) Acid value : not more than 2·0.
(iv) Saponification value : 188–195.
(v) Iodine value : 79–85.
(vi) Unsaponifiable matter : 0·8–1·3% (iodine value of unsaponifiable matter not less than 185).
(vii) (*a*) After passage of a current of air at 15–20° C. for three days, the oil, on addition of an ethereal solution of phloroglucinol with a few drops of hydrochloric acid, gives not more than a faint pink coloration (Kreis test for rancidity). The fresh oil should not give the faintest coloration in this test.
(*b*) The peroxide value (Lea test) of the fresh oil should not exceed 1 and, after passage of a current of air at 15–20° C. for three days, should not have risen to more than 10.

A. The need for a simultaneous test on the Standard of reference whenever a determination of vitamin E is made.

In the collaborative experiment mentioned above, the workers were supplied with 4 solutions of the sample of synthetic α-tocopheryl acetate which was ultimately adopted as the International Standard for vitamin E. The strengths of the four solutions were graded 8 : 4 : 2 : 1 but no indication as to which solution was which was given. Workers were asked to determine the relative potencies of the solutions. When the identity of the various solutions was disclosed, curves of response relating percentage of positive responses to the logarithm of the dose given were drawn. The median fertility dose, *i.e.* the dose which brought about 50% of positive responses for each laboratory was calculated. It varied from 0·55mg. in one laboratory to 1.71mg. in another laboratory (Table IX). This is very clear evidence of the need of a Standard of Reference for work in different laboratories. The writer has not been able to obtain a series of results from the use of any one preparation of vitamin E in any one laboratory, but it is very probable indeed that the variation in response to the same dose of vitamin E would be

similar to that obtained for other vitamins, and it must be urged that a simultaneous test on the Standard should always be made whenever a determination of vitamin E is made.

TABLE IX

EVIDENCE THAT A SIMULTANEOUS TEST OF THE STANDARD FOR VITAMIN E SHOULD BE MADE FOR COMPARISON OF RESULTS OBTAINED IN DIFFERENT LABORATORIES. FROM THE "INTERNATIONAL STANDARD FOR VITAMIN E," BULL. HEALTH ORG., LEAGUE OF NATIONS 1940–41, 9, 436.

Laboratory.	Number of rats used.	Median fertility dose (mg.).
1	83	0·56
2(a)	40	0·55
(b)	42	0·66
3	91	0·66
4	68	0·72
5	48	0·84
6(a)	79	1·13
(b)	50	1·14
7	78	1·36
8(a)	52	1·50
(b)	52	1·05
9	58	1·71

B. The dilution of the Standard of reference for dosing.

The solution of the Standard in olive oil has been found by spectrophotometric tests to be very stable, but it should be kept at 0° C. or lower and removed only when dilutions for feeding are to be made. The olive oil used for dilution should conform to the requirements set out above and the dilutions should be of such strength that the dose to be given to a rat should be contained in an amount of oil conveniently given orally from an Agla micrometer syringe fitted with a blunted injection needle. This should be manipulated as described under vitamin D and allowance made for the specific gravity of the oil in calculating the volume of solution which contains the weight of vitamin E required for the dose. The dilutions should be made up once a week and kept in the cold store.

C. The general arrangement of the test for a determination of the vitamin E potency of a substance in terms of the International Standard.

This should follow the arrangement suggested for each of the other vitamins. Three doses of the Standard in the ratio of

4 : 2 : 1 (say 2mg., 1mg., 0·5mg.) should be tested on three groups of about ten rats each, and two or three doses of the substance under test also in the ratio of 2 : 1 or 4 : 2 : 1 should be tested on two or three other groups of about 10 rats each, the object being to find two or three doses of the test substance which give results similar to those given by comparable doses of the Standard. It is highly desirable that one or two groups should give more and one or two groups less than 50% of positive responses for both the Standard and the test substance, since the best comparison is made by comparing the doses of substance and Standard respectively which bring about 50% of positive responses, called in this case the median fertility dose.

2. The Preparation of Rats for a Determination of Vitamin E

A. Animals suitable for the test.

Young rats are raised from weaning to adult size on a diet free from vitamin E. Some workers then mate their young does and only when they have undergone at least one gestation-resorption, use them for vitamin E tests. Bacharach, Allchorne and Glynn (1937), however, tried using virgin rats for the tests, as well as others which had undergone gestation-resorption and they obtained reliable evidence not only that there was a probability approaching certainty that their E-free diet would produce a gestation-resorption in their virgin does, but that a higher proportion of virgin does showed implantation after mating than of does which had had one gestation-resorption or more. Since only those does which have shown the sign of implantation (blood in the vagina on the 13th–15th days) are the ones from which the percentage of positive results (birth of a litter) is to be calculated, it follows that much time and labour and animals also may be saved by using virgin does. Naturally, a worker would include a group of does receiving no dose of vitamin E in every test carried out until he was well satisfied that the animals of his colony behaved as well as do those of Bacharach's. Rats should not be used more than once for a vitamin E determination, as there is no means of knowing whether the whole of the dose given the first time has been used up. Rats should be not less than 3 months and not more than 5 months old when mated.

B. Housing of the animals.

Rats may be kept in the ordinary cages of the colony until dosed, when each doe should be given a separate cage, and a few days before parturition a grid of ¾″ or 1″ mesh to cover the whole floor of the cage and stand 2″–2½″ above it. This allows the young to fall through it, out of reach of the doe who might otherwise devour dead ones. (See paragraph on Criteria.)

C. Diet.

The diet used by Evans and Burr (1927) in their work on vitamin E consisted of :

Casein	18 parts.
Corn Maize starch	54 ,,
Lard	15 ,,
Milk-fat	9 ,,
Salts	4 ,,
	100 ,,

In addition each rat was given 0·4–0·6g. dried yeast daily.

Rowlands and Singer (1936) and Bacharach, Allchorne and Glynn (1937) used the following diet :

Casein	18 parts.
Rice starch	49 ,,
Lard	12 ,,
Dried yeast	10 ,,
Cane sugar	4 ,,
Salt mixture	5 ,,
Cod liver oil	2 ,,
	100 ,,

Coward and Morgan (unpublished results) have also used the diet in their laboratory but to a more limited extent than Bacharach. However, gestation resorption occurred in every one of 32 does which had been fed on this diet only since weaning, mated with a buck of proven fertility and had shown the sign of implantation (blood in the vagina on the 14th day of pregnancy).

D. Dosing the rats.

The total dose to be given to a rat should be spread evenly over the first five days after positive mating. The worker must be prepared to find that some of the rats of every group do not show the sign of implantation (blood in the vagina on the 14th day of pregnancy). This has nothing to do with vitamin E and these rats must be thrown out of the test. It is the rats which have shown the sign of implantation which form the basis for

calculating the percentage of positive results (production of a litter). Allowance must be made for this in preparing for a vitamin E determination; more rats must be brought up on the basal diet and more than the desired number of rats must be assigned to each group and dosed. An Agla micrometer syringe fitted with a blunt injection needle is strongly recommended for dosing the rats.

In the last few years it has been suggested that there is some interrelationship between vitamin A and E in the animal body (see Chapters III and VIII). It even seems that vitamin E may act as an antioxidant to vitamn A when the two are given to the rat about the same time. If that is so, then vitamin E *may* lose activity to the extent to which it preserves vitamin A activity. This has not been demonstrated but it would probably be good practice to withhold the cod liver oil from the diet of the rat during the period of dosing with vitamin E and for one day before and after this period.

3. Criteria for the Measurement of the Response of Rats to Doses of Vitamin E

There is no evidence at all that the size of a litter is dependent on the dose of vitamin E given. Moreover, non-viable young may be eaten by the doe and thus not allowed to fall through the grid of the cage. They would therefore be missed in assessing the number of young born. Hence the response to vitamin E is best considered as an "all or none" reaction and the birth of a litter of one or more rats, alive or dead, as a positive response to a dose of vitamin E. Correlated with this is a steady increase in weight of the doe from about the 12th to the 21st day of pregnancy, when a sudden drop in weight would indicate the birth of a litter even if a rat had eaten all its young. In a gestation-resorption the weight of the doe increases to about the 16th day and then gradually falls to about its weight at mating by the 21st day.

The curve of response to doses of vitamin E.—The "fertility rate" is taken as the measure of response, *i.e.* the number of litters born expressed as a percentage of the number of does which showed the implantation sign. When these percentage responses or fertility rates are plotted against the logs. of the doses given, an S-shaped or sigmoid curve of response is obtained.

Working out a result.—Suppose the following results had been obtained in a determination of the vitamin E content of a foodstuff, and that equal numbers of rats had been used in all doses.

	Dose.	% positive responses.
Group 1	0·8mg. Inter. St.	5
,, 2	1·2 ,, ,,	40
,, 3	1·8 ,, ,,	70
,, 4	2·7 ,, ,,	90
,, 5	0·5g. subs. X	10
,, 6	1·5 ,, ,,	55
,, 7	No dose.	0 (not used in the calculation).

The calculation proceeds as in the pigeon test for vitamin B_1, which depends on the fact that if the normal equivalent deviation or probit of the percentage of positive responses is plotted against the log. of the dose given, a straight line is obtained. The average slope of the two lines from this test is calculated, lines with this average slope drawn through the "mid-points" of the curves and the horizontal distance between the parallel lines determined. This is the log. of the ratio of the potencies of substance X and the Standard.

% of positive responses.	Normal equiv. deviation, y	Dose.	Log. dose, x	$x-\bar{x}$	$y(x-\bar{x})$	$(x-\bar{x})^2$
5	−1·6449	0·8mg. I. St.	$\bar{1}$·90309	−0·264135	+0·434475	·069767
40	−0·2533	1·2mg. ,,	0·07918	−0·088045	+0·022302	·007752
70	+0·5244	1·8mg. ,,	0·25527	+0·088045	+0·046171	·007752
90	+1·2816	2·7mg. ,,	0·43136	+0·264135	+0·338515	·069767
	4) −0·0922		4)0·66890		Σ=0·841463	Σ=0·155038
	$\bar{y}$= −0·02305		$\bar{x}$=0·167225			

$$b=\frac{0\cdot841463}{0\cdot155038}=5\cdot4275$$

and $y=\bar{y}+5\cdot4275(x-\bar{x})$

$=-0\cdot02305+5\cdot4275x-0\cdot90761$

$=5\cdot4275x-0\cdot9307$

For checking the curve.

x	y
0	−·93
·4	+1·24

% of positive responses.	Normal equivalent deviation.	Dose.	Log. dose.
10	−1·2816	0·5g. X	$\bar{1}$·69897
55	+0·1257	1·5g. X	0·17609
	Diff.=+1·4073		Diff.= ·47712

$$b=\frac{1{\cdot}4073}{{\cdot}47712}=2{\cdot}9496$$

The average slope of the two curves is

$$\frac{\overset{*}{2}\times5{\cdot}4275+\overset{*}{1}\times2{\cdot}9496}{3}=4{\cdot}6015$$

The point with average x and y values in the curve for the Standard is 0·1672, −0·0230. The point with average x and y values in the curve for the substance X is $\bar{1}$·9375, −0·5779. The curve with average slope through "mid-point" of the curve for the Standard is

$$\frac{y-(-0{\cdot}0230)}{x-0{\cdot}1672}=4{\cdot}6015$$

therefore $$y=4{\cdot}6015x-0{\cdot}7924$$

The curve with average slope through "mid-point" of the curve for substance X is

$$\frac{y-(-0{\cdot}5779)}{x-\bar{1}{\cdot}9375}=4{\cdot}6015$$

therefore $$y=4{\cdot}6015x-0{\cdot}2903$$

The horizontal distance between these two parallel lines is given by

$$\frac{y}{x}=4{\cdot}6015$$

therefore $$\frac{-0{\cdot}2903-(-0{\cdot}7924)}{4{\cdot}6015}=x=0{\cdot}1091=\text{log. }1{\cdot}2856$$

Therefore the potency of grams of X : mg. International Standard, is 1·2856, *i.e.* substance X contains 1·2856 or say, 1·3mg. of the International Standard, or 1·3 International units of vitamin E per gram.

N.B. In this calculation, logs. of *mg.* of International Standard and logs. of *g.* of substance X were used in order to save a little

* To allow for twice as many rats having been used for the Standard as for the Substance X.

arithmetic, but this had to be remembered in the last line of the calculation.

(See also Chapter XI.)

The student is recommended to work out the whole of this example starting with, and keeping to, only 3 figures in the decimals throughout, in order to see how far this exact arithmetic is worth while.

He is also strongly urged to check each curve as soon as it is calculated, by substituting simple values for x, finding the corresponding values for y and drawing out the curves on graph paper.

Other criteria for the measurement of the response to vitamin E.—Many workers have tried to find a criterion which would give a response graded to the dose of vitamin E given, *e.g.* the number of young delivered at term, but even when grids of wire mesh are provided, it cannot be certain that a doe has not eaten a part of her litter. Hence the data obtainable for this criterion are not reliable and should not be used.

Mason (1942) as the result of a very extensive series of experiments has evolved the following method of measuring the response to doses of vitamin E. On the 16th day of pregnancy, the doe is killed, the cervix severed at its "mid-point", and the uterus with its contents freed from mesentery and ovaries. The numbers of live fœtuses (N), dead fœtuses and resorptions are recorded, the uterus emptied and weighed. The difference gives the weight of the uterine content (W in grams). If only one viable fœtus is found, it is ignored and $N=0$, but if more than one viable fœtus occurs, $N=$the whole number. (Mason considered that a single live fœtus at the 16th day was unlikely to survive till the 21st). The formula proposed is $\frac{W+N}{5}=$uterine index. Values of 1 or more are regarded as positive, those lying between 0·35 and 0·99 are considered subminimal, and those of 0·35 and less are considered to be negative. The responses greater than 1·0 range from 1 to 4 and have been found in general to be proportional to the dose given. Except for the recognition of the subminimal type of response Mason considers his method not much superior to that based on the "all or none" reaction. It does, however, shorten the test period of the assay and prevents the misinterpretation of certain atypical uterine states as negative responses to dose.

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CHAPTER VIII

THE INTERDEPENDENCE OF THE VITAMINS

IT is well known that when young rats are given a diet deficient in vitamin A, they eventually lose weight and die, no matter how much of the other vitamins may have been given to them throughout the experiment. Rats also die if their diet contains abundance of all the vitamins except vitamin B_1. Guinea-pigs die if their diet contains abundance of all the vitamins except vitamin C and rats die in time if their diet contains no vitamin D, even though it contains all the mineral elements in the most satisfactory proportions known. Thus a complete deficiency of any one vitamin cannot be rectified by giving an abundance of the other vitamins.

1. The Influence of a Partial Deficiency of one Vitamin in the Basal Diet on the Determination of another Vitamin

There is a certain amount of evidence in the literature that when the supples of one vitamin are limited the effect of giving a small dose of a "test" vitamin is less than the effect would have been if the supplies of the other vitamins had been larger throughout. Consequently a larger dose of the "test" vitamin has to be given in order to bring about a result equal to the result that a small dose of "test" vitamin would have given in the presence of large supplies of the first vitamin. Perhaps the best way to demonstrate this point is to describe the particular experiments which have shown it.

A. Early evidence of the influence of a partial deficiency of one vitamin on the determination of another vitamin.

Sherman and Munsell (1925) published a series of results from groups of rats which, having ceased to grow on a diet deficient in vitamin A, were given graded doses, 0, 0·05, 0·1, 0·2, 0·5, 1·0 and 2·0g., respectively, of tomato, every rat of any one group being given the same dose. The experiment was carried on for 8 weeks. It produced a series of composite growth curves whose slopes were nicely graded to the doses of tomato given. Later, Sherman and Batchelder (1931) published another series of composite growth curves of groups of rats, similarly prepared, but given graded doses of dried whole milk (the ratios only of the doses being stated, 0, 1X, 2X, 4X, 8X and 16X). This experiment was carried on for 8 weeks also, and again a nicely graded series of growth curves was obtained; but it was at once evident that the "spread" of the curves obtained in response to doses of milk was greater than that obtained in response to doses of tomato. Sherman and Batchelder say in their paper: "From the view-point of present-day knowledge, however, it is readily conceivable that the less accentuated response to increasing levels of feeding of the tomato may have been because this was a less adequate source than milk of some factor or factors whose influence upon growth was not fully realised at the time of the earlier experiments or possibly that, notwithstanding the general equivalence of the vitamin A of animal origin and its precursor

of vegetable origin, there may still be a difference in the degree of readiness and completeness of utilisation of the two forms which might become measurable in the averages of sufficiently numerous experiments made in as rigorously quantitative a way as our present knowledge and experience permit." It should here be pointed out, however, that in one respect the two experiments differed. In the experiment in which different doses of tomato were give, the basal diet of the rats contained only 5% of dried brewers' yeast, whereas in the experiment in which different doses of dried milk were given the basal diet contained 10% of dried brewers' yeast. This difference may account for the difference in the "spread" of the composite growth curves obtained in the two experiments, for similar differences in the "spread" of results have been obtained by the writer by varying the amounts of one of the vitamin constituents of the basal diet.

B. The response to doses of vitamin D when the supplies of vitamin A are limited.

In 1932, Coward, Key and Morgan published an account of an attempt to determine vitamin D by an "increase in weight" method arranged like the "increase in weight" method for the determination of vitamin A.

The basal diet consisted of:

Caseinogen, "light, white" (B.D.H.)	15%
Dextrinised rice starch	73%
Dried yeast	8%
Salt mixture (Steenbock's 40)	4%

The salt mixture consisted of:

Sodium chloride, NaCl	23·4g.
Magnesium sulphate, $MgSO_4.7H_2O$	24·6g.
Disodium hydrogen phosphate, $Na_2HPO_4.12H_2O$ ..	35·8g.
Dipotassium hydrogen phosphate, K_2HPO_4	69·6g.
Calcium phosphate, $Ca_2H_2(PO_4)_2.4H_2O$	68·8g.
Calcium lactate.$5H_2O$	15·4g.
Iron citrate.$6H_2O$	6·0g.
Potassium iodide, KI	0·2g.

In addition each rat was given a daily dose of 0·04mg. of the light petroleum-soluble fraction of dried carrots as a source of vitamin A. The carrots were minced, spread in a very thin layer on sheets of glass, and dried in a current of air at a temperature of 50–60°. The dried material was then extracted twice with light petroleum and the extract evaporated on a warm sand

bath in a current of nitrogen. The oily residue was diluted with olive oil to such a concentration that the required amount was contained in 0·02g. oil which was given daily as one drop directly into each rat's mouth. Fresh supplies of this preparation were made about twice a week. It was not realised at the time that vitamin A probably exists only as the provitamin, carotene, in plant tissues. The dose of unsaponifiable matter contained only about 0·2μg. carotene, which is now known to be much below the daily requirement of the growing rat.

The experiment was carried out as described in Chapter VI. Briefly, the rats were given the basal diet plus the carrot extract until they ceased to grow. As they became steady in weight they were divided into seven groups. The rats of the different groups were given daily doses of 0, 0·01, 0·02, 0·05, 0·1, 0·5 and 1·0 unit of vitamin D, each rat of any one group being given the same dose for 5 weeks. The average increases in weight of the

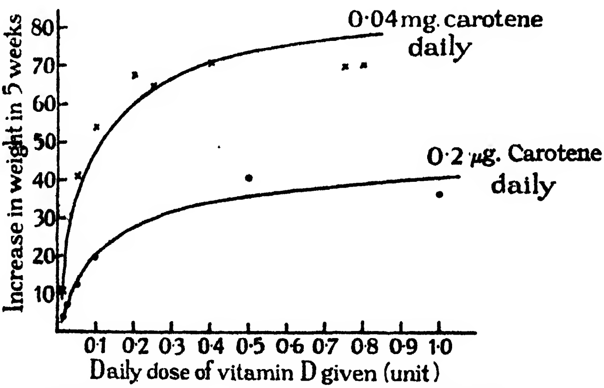

FIG. 30.—The influence of the vitamin A content of the diet on the response to doses of vitamin D.

different groups were calculated and plotted against the doses of vitamin D given. A fairly smooth curve of response was obtained. It was, however, decided to determine two more points in the curve from doses of 0·25 and 0·75 unit of vitamin D. Further groups were then formed of rats which had been prepared similarly except that, to economise labour and time, a commercial preparation of carotene (B.D.H.) was used instead of the crude carrot extract prepared in the laboratory. The same weight of carotene as of extract was used for it had been considered that 0·04mg. carrot extract had supplied abundance of vitamin A,

and if the same weight of carotene was given that also would supply abundance of vitamin A. The average increases in weight in 5 weeks of these two groups of rats were found, but when applied to the graph of the result from the other groups of rats, they were found not to lie on the curve of response at all, but far above it. As the source and probably the amount of vitamin A was the only factor known to be different in the two experiments, it was decided to test other doses of vitamin D on rats given 0·04mg. carotene instead of carrot extract. The doses then tested were 0·01, 0·05, 0·1, 0·2, 0·4, 0·8 and 10·0 International units, the last being considered a massive dose. Thus altogether nine doses of vitamin D were tested with 0·04gm. carotene as a source of vitamin A. The average increases in weight of the nine groups of rats when plotted against the doses of vitamin D given formed a curve of response which was fairly smooth considering the small number of animals in each group. This curve was decidedly steeper than the one obtained when 0·04mg. carrot extract containing only 0·2μg. carotene had been given as the source of vitamin A (Fig. 30). This was strongly suggestive that the response to one vitamin is influenced by the amount of another vitamin available.

C. The response to doses of vitamin B_1 when the supplies of one or more of the other vitamins B (or some other constituent of "autoclaved yeast") are limited.

Just as Coward, Key and Morgan had found that inadequate supplies of vitamin A in the basal diet modified the response of rats to graded doses of vitamin D, so Coward, Burn, Ling and Morgan (1933) found that inadequate supplies of autoclaved yeast, shown by tests on pigeons to contain no vitamin B_1, modified the response of rats to graded doses of vitamin B_1. In a first attempt to construct a curve of response to graded doses of vitamin B_1 the following basal diet was used :

Caseinogen, light white (B.D.H.)	15%
Dextrinised rice starch	71%
Agar-agar	2%
Salt mixture (Steenbock's 40) (see p. 155)	4%
Dried brewer's yeast autoclaved at 120° for 6 hours, dried at 100° C. overnight and ground finely	8%

In addition each rat was given 0·1g. (5 drops) of a good sample of cod liver oil twice a week to supply vitamins A and D.

The brewers' yeast, after autoclaving, did not contain enough vitamin B_1 to cure retracted neck in any of 3 pigeons when a dose of 1·0g. was administered, whereas a dose of 0·03g. of the International Standard has cured in twenty-one tests from 33 to 86%, and a dose of 0·1g. of different samples of dried yeast (non-autoclaved) has cured from 10 to 87% of the birds used in each test. It was therefore considered to contain negligible quantities of vitamin B_1, if any.

The rats were prepared as described in Chapter IV. Briefly, they were given a preparatory period of 10–14 days when no supplement but cod liver oil was given and they became steady in weight. They were then divided into six groups as nearly comparable as possible regarding weight, sex and litter. Each rat was given a separate cage, with a grid of $\frac{1}{2}''$ mesh. The groups were given daily doses of 0, 0·005, 0·02, 0·04 and 0·1g. respectively of the International Standard for 3 weeks, every rat of any one group being given the same dose.

The average increases in weight of the different groups were calculated and plotted against the dose of Standard given (Fig. 31). A curvilinear relationship was obtained which looked satisfactory until other results obtained at the same time were applied to the curve and it was found that the curve was useless for interpreting these results. Two doses 0·1 and 0·3g. of a certain sample of wheat embryo had been found to produce average increases of −1·12 and +33·37g. respectively in 3 weeks. The abscissa of the curve corresponding to an increase in weight of −1·12g. was found to be 0·008. Therefore the increase in weight produced by giving 0·3g. wheat embryo should have a corresponding abscissa of $3 \times 0{\cdot}008$, *i.e.* 0·024. Actually the increase in weight of 33·37g. from 0·3g. wheat embryo did not fall on the curve at all; the curve had flattened without reaching that point, and the increase in weight corresponding to abscissa 0·024 was 20·25g. Similarly, two doses of 0·05 and 0·2g. dried yeast tested at the same time had given increases in weight in 3 weeks of −2·65 and 42·85g. respectively. The abscissa of the curve corresponding to an increase in weight of −2·65g. was 0·0075. Therefore the increase in weight from 0·2g. dried yeast should have a corresponding abscissa of $4 \times 0{\cdot}0075$, *i.e.* 0·03. Actually the increase in weight of 42·85g. for 0·2g. dried yeast did not fall on the curve at all. Again, two doses of 1·0 and 3·0g. of a certain food preparation also tested at the same time produced increases in weight in 3

weeks of 23·5 and 46·45g. respectively. The abscissa of the curve corresponding to an increase in weight of 23·5g. was 0·03. Therefore the increase in weight from 3·0g. of the food should have a corresponding abscissa of 3×0·03, *i.e.* 0·09, but again the actual increase in weight was too high to fall on the curve (Fig. 31). It therefore seemed probable that these test substances were supplying some factor necessary for growth which had not been supplied in sufficient amount to the rats used for the construction

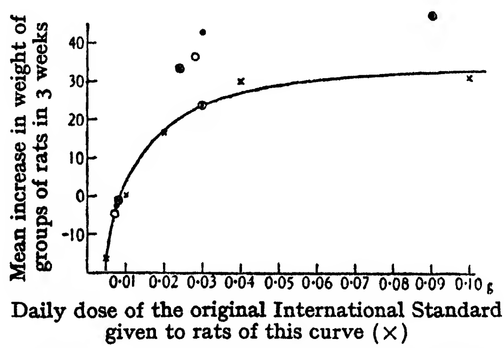

FIG. 31.—A curve of response to doses of the International Standard for vitamin B_1, obtained when only 8% autoclaved yeast was put in the basal diet. It was useless for determining the vitamin B_1 content of other substances, for the higher doses of these other substances produced greater increases in weight than any produced by doses of the Standard.

× = Mean increases in weight of the groups of rats given graded doses of the Standard.

• = Mean increases in weight of groups of rats given daily 0·05 and 0·2g. respectively of a sample of dried yeast I.

⊙ = Mean increases in weight of groups of rats given daily 1·0 and 3·0g. respectively of a commercial food sample.

⊗ = Mean increases in weight of groups of rats given daily 0·1 and 0·3g. respectively of a sample of wheat embryo.

O = Mean increases in weight of groups of rats given daily 0·05 and 0·2g. respectively of yeast extract I.

The result from the lower dose of each test was plotted on the curve itself. The result from the higher dose of each test was plotted against the abscissa corresponding to the appropriate multiple of the abscissa found for the lower dose.

of the curve, either in their basal diet or in the different supplements of the International Standard for vitamin B_1. It seemed possible that the autoclaving of the yeast had destroyed not only the vitamin B_1 but also some other factor which is necessary for growth. Chick and Roscoe (1927) showed that when brewers' yeast is heated at 120° C. for 5 hours at its normal *p*H of about 4·5–5·0 it loses half of its vitamin B_2 potency. Williams, Waterman and Gurin (1929) obtained similar results. It was therefore

concluded that although 8% of unheated dried yeast provided ample amounts of all the vitamins B, yet 8% of autoclaved yeast was seriously deficient in vitamin B_2 and possibly in other factors also.

Therefore a new curve of response to doses of 0·005, 0·01, 0·02, 0·04 and 0·1g. of the original International Standard for vitamin B_1 was constructed in the same way as the last, except that the

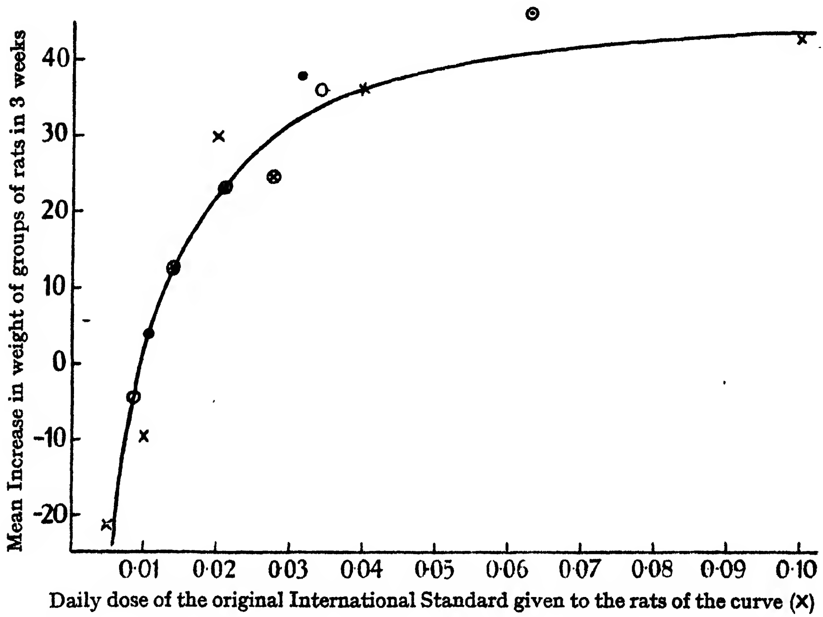

FIG. 32.—A curve of response to doses of the International Standard for vitamin B_1 obtained when 20% autoclaved yeast was put in the basal diet. It could be used for determining the vitamin B_1 potency of other substances, for it was steeper than the one obtained with 8% autoclaved yeast. Presumably 20% A.Y. supplied abundance of the vitamins other than B_1, whereas 8% did not.

Interpretation of symbols as for Fig. 31.

amount of autoclaved yeast (again heated at 120° for 6 hours) was raised from 8 to 20%. The food substances were not retested. The new curve of response (Fig. 32) was much steeper than the former one, and, when the original results from the food substances were applied to it, the actual results were found to be in very good agreement with those calculated by the method described in connection with the first curve (Table X).

As the three substances tested on the curve were of very varied

nature (wheat embryo, dried yeast and a more bulky food preparation) and the pairs of doses of each gave concordant results when compared by means of the curve, it was therefore concluded that the second diet provided ample supplies of all growth-promoting factors for vitamin B_1 tests. Additional support of the validity of the second curve was obtained by testing pairs of doses of three other substances with 20% autoclaved yeast in the basal diet. In all of these the apparent ratio of the potencies of the two doses of a pair, determined from the curve as the ratio of the abscissæ corresponding to the mean increases in weight of the groups of rats used in the tests, was very nearly equal to the known ratio of the doses (Table X).

TABLE X

Substance tested.	Dose, g.	No. of rats.	Mean increase in weight,* g.	Abscissa corresponding to mean increase in weight.	Apparent ratio of doses.	Actual ratio of doses.	Slope of curve = Inc. in wt. ÷ (log. x_1 − log. x_2)
Dried yeast I	0·05	10	−2·65	0·0096	1 : 4·2	1 : 4	75·57
	0·2	10	42·85	0·0407			
Food substance	1·0	6	23·50	0·0226	1 : 2·1	1 : 3	48·10
	3·0	6	46·45	0·0467			
Wheat embryo	0·1	6	−1·12	0·0101	1 : 3·5	1 : 3	72·29
	0·3	6	33·37	0·0352			
Yeast extract I	0·05	5	−4·60	0·0090	1 : 3·8	1 : 4	67·68
	0·2	5	36·15	0·0341			
Dried yeast II	0·05	3	4·2	0·0125	1 : 2·9	1 : 3	70·84
	0·15	4	38·0	0·0365			
Yeast extract II	0·1	4	12·8	0·0160	1 : 1·6	1 : 2	38·87
	0·2	4	24·5	0·0255			

* The numbers of bucks and does in each group were not always equal. Hence, the average results from bucks and does separately were calculated and then the mean of the two averages calculated, so that each average was virtually obtained from an equal number of bucks and does and could therefore be used as an "average buck and doe" curve.

The difference in slopes of the two curves of response to doses of vitamin B_1 obtained by giving different amounts of autoclaved yeast in the basal diet demonstrates afresh that the response of animals to doses of one vitamin is influenced by the amount of another vitamin supplied.

Nowadays, very little use is made of a general curve of response to graded doses of a vitamin, however well determined it may be. The slope used in a determination of the vitamin content of a substance is the average of the two slopes obtained in that test from two (or more) doses of the Standard and two (or more) doses of each substance being tested. The slopes obtained from two doses of each substance in Table X have been added to show how much they may vary.

D. The response to doses of vitamin A when the supplies of a certain unknown factor (possibly a vitamin) are limited.

From time to time Coward and co-workers (1929, 1 and 2, 1930, 1931) have published evidence that a particular kind of caseinogen (light white B.D.H.) possesses greater growth-promoting power than another kind of casein supplied by another commercial firm for purposes of vitamin-testing. Whether the factor is a vitamin or some other factor necessary for growth has never been determined, but certain points in the work suggest that the factor may be a vitamin. Work by Mapson (1932, 1933) suggests that a substance recognised by him in liver and certain liver extracts may actually be the same factor that Coward found associated with caseinogen (and various other substances also, *e.g.* liver, wheat embryo, milk, etc.). In the discussions of papers at the meeting of the American Institute of Nutrition held in Washington, 1936, other workers reported the existence of some substances which may prove to be identical with what has been spoken of in the literature as the "casein" factor (whatever that may be).

This factor is evidently necessary for growth and should therefore be present in abundance in all diets used in "increase in weight" methods. In a paper by Coward, Key, Dyer and Morgan (1930) are described the results of an attempt to construct a curve of response to graded doses of vitamin A when 15% of the "Glaxo" casein was incorporated in the diet as the source of protein. The average increases in weight of the groups of animals given graded doses of a certain sample of cod liver oil were published, but no curve relating mean increase in weight to dose of vitamin A (cod liver oil) given. It is given here, however (Fig. 33), to show graphically what a very shallow and useless curve it was. Recognising the uselessness of such a curve and recognising also that some constituent of the diet must be faulty,

Coward, being familiar with Palmer's work on the preparation of different forms of casein, immediately suspected the casein of being somehow inadequate. She then constructed a fresh curve of response to graded doses of vitamin A, using the same sample of cod liver oil as in the construction of the first curve, and using as a source of protein the caseinogen sold by the British Drug Houses under the name "light white". A very different curve was obtained (Fig. 33); it was much steeper than the first curve and also much smoother. (The greater smoothness of the curve *may* be accounted for by supposing that differences in the different rats' reserves of the "casein factor" were

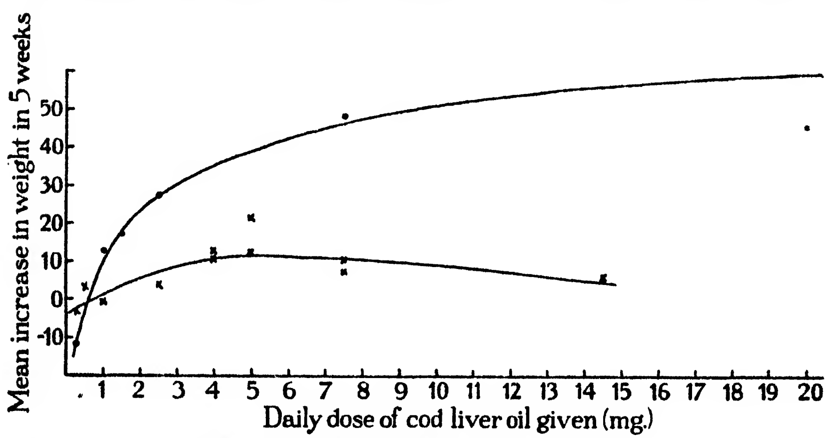

FIG. 33.—Two curves of response relating increase in weight in 5 weeks to doses of the same sample of cod liver oil given, obtained at the same laboratory. The steeper one was constructed when light white (B.H.D.) casein was used as the source of protein, the less steep one when Glaxo "vitamin-free" casein was used.

made good by the casein used in the construction of the second curve but not by the casein used in the first.)

Whatever may prove to be the difference between the two forms of casein, the fact remains that different curves of response to doses of vitamin A can be obtained by varying one constituent of the basal diet. In order to be sure that a basal diet contains all growth-promoting substances in ample amounts, the International Standard and different substances, as much varied in nature as possible, should be tested, each in two or more doses.

The slopes of the curves of response should be compared and if any of those obtained with food stuffs are significantly different from that obtained with the International Standard, then there

is good reason for thinking that the basal diet is lacking in some factor which the foodstuffs have supplied, and it should be carefully examined.

E. The response to doses of vitamin A when the supplies of vitamin E are limited.

Hickman and his colleagues (1944) have demonstrated a dependence of vitamin A on vitamin E in its manifestation by increase in weight of the rat. Their procedure was, briefly, to give young rats a diet containing no vitamin A and only a small amount of vitamin E, then to give all the rats a small daily dose of vitamin A, and having divided them into different groups, to give graded doses of vitamin E to the various groups. After five weeks the composite growth curves of the groups were plotted against time. The curves were nearly parallel, and, within limits, the rats receiving the higher doses gave the higher growth curves. A very similar result was obtained when β-carotene was used in place of the crystalline vitamin A. Hickman speaks of the "sparing action" of vitamin E on vitamin A or carotene and suggests that it is due chiefly to repression of oxidation in and near the gastrointestinal tract.

2. The Interdependence of the Vitamins

All this work has demonstrated the influence of one vitamin on the activity of another, but by methods which R. A. Fisher (1942) would surely call clumsy and extravagant. A well-designed experiment can give more information and more accurate information at less expenditure of animals, diet, space, labour and time, than an experiment which is designed to test one effect only. Thus, knowing that vitamin E is essential for growth (Evans, 1928), Hickman's experiments could have been planned not only to show the influence of vitamin E on the activity of vitamin A, but also to show the influence of vitamin A on the activity of vitamin E. This could also have been extended to the influence of both vitamins on reproduction as well as to their influence on growth.

A very simple experiment (perhaps unnecessarily simple) has just been completed in the writer's laboratory which demonstrates this point. Vitamin B_1 and riboflavin are both necessary

for the growth of the rat. Early experiments had demonstrated that when the amount of "vitamin B_2" in the diet was limited, the response to graded doses of vitamin B_1 was limited also, not only at the upper levels of dosage as might have been expected, but at the lower levels also. This was a good indication of the dependence of one vitamin on another, but obviously the next step was to carry out an experiment on the lines suggested by Fisher, which would investigate simultaneously the interdependence of the two vitamins on each other. Eighty rats were selected from litters containing 4 males or 4 females each. They were arranged in 4 groups, the 4 rats of each litter being evenly distributed between the 4 groups. Each group contained 10 males and 10 females. Each rat was housed in a separate cage provided with a grid of ½″ mesh. The diet of the rats consisted of:

Casein (Glaxo, ashless extracted)	15%
Rice starch partially dextrinised	81%
Salt mixture (Steenbock's 40)	4%

Five drops of a good sample of cod liver per rat twice a week.

0·5ml. liver extract, norite treated, equivalent to 5g. whole liver per day, per rat.

The respective groups were given daily from the beginning of the experiment:

Group 1	1 I.U. Vitamin B_1	2·5μg. riboflavin
,, 2	2 ,, ,,	2·5μg. ,,
,, 3	1 ,, ,,	5·0μg. ,,
,, 4	2 ,, ,,	5·0μg. ,,

The test was carried on for five weeks.

The rats were weighed once a week. At the end of that time, the increases in weight of the rats in each group were averaged. The averages of male and female rats in each group differed only slightly and not always in the same direction, so they were not treated separately in the calculations. The averages (g.) may be plotted thus:

	1 I. U. B_1	2 I. U. B_1
2.5 Rib.	24·85	29·25
5·0 Rib.	29·70	35·40

The result may be stated simply as:

1. Doubling the dose of vitamin B_1 had some effect but it had a greater effect when the dose of riboflavin was also doubled.
2. Doubling the dose of riboflavin had some effect but it had a greater effect when the dose of vitamin B_1 was also doubled.

Thus the interdependence of two vitamins is demonstrated simultaneously in one experiment. For further information obtainable from this experiment, see Chapter XII.

3. The Influence of an Excess of one Vitamin in the Basal Diet on the Determination of another Vitamin

After demonstrating the influence of a partial deficiency of one vitamin in the basal diet used on the determination of another vitamin, it seemed necessary to investigate the influence of an excess of one vitamin on the determination of another. An additional reason for making this investigation was the fact that when Culhane and Coward compared two particular samples of cod liver oil they obtained the same ratio for the potencies (3 : 1), yet the average responses of Culhanes' groups of rats given certain doses were only about half of the average responses of Cowards' groups of rats given the same doses. A comparison of the basal diets used by the two workers showed two main differences. Coward had used light white (B.D.H.) casein whereas Culhane had used a strongly heated casein. Moreover, Coward had given only 8 International units of vitamin D per week to each rat and Culhane had given 200 International units. The first of these differences was thought to be the probable cause of the differences in response, but the other difference seemed to be worth investigating.

A. The influence of an excess of vitamin D on the determination of vitamin A.

Bruce and Phillips (1938) prepared rats as for a vitamin A determination, giving them the vitamin A-free diet described in Chapter III with the usual supplement of 8 International units of vitamin D per rat per week. As they became steady in weight they were divided into three groups which were given 8, 25 or

100 units respectively of vitamin D per rat per week. All the rats of all three groups were given 2mg. cod liver oil each, per day, for 4 weeks. The amount was reduced after the first week because it appeared to be a rather high dose. The result may be seen in Table XI.

TABLE XI

TO SHOW THAT EXCESS OF VITAMIN D DOES NOT INFLUENCE THE RESPONSE TO A DOSE OF VITAMIN A. DIFFERENT GROUPS OF RATS WERE GIVEN WEEKLY DOSES OF 8, 25 AND 100 UNITS RESPECTIVELY OF VITAMIN D FOR 5 WEEKS AFTER THEY HAD BECOME STEADY IN WEIGHT ON A VITAMIN A-FREE DIET SUPPLEMENTED BY 8 UNITS OF VITAMIN D PER WEEK. ALL WERE GIVEN A DAILY DOSE OF 2MG. COD LIVER OIL FOR 7 DAYS AND THEN A DAILY DOSE OF 1MG. COD LIVER OIL FOR 28 DAYS.

	No. of rats in group.		Average weight at beginning of prep. period, g.		Average weight when steady, g.		Average weight after dosing with cod liver oil, g.		Average increase in weight after 5 weeks, g.	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Rats given 8 International units of vitamin D per week	4	10	27·7	26·0	64·5	72·6	83·5	90·7	19·0	18·1
Rats given 25 International units of vitamin D per week	6	11	28·7	28·4	76·0	72·6	94·0	90·5	18·0	17·8
Rats given 100 International units of vitamin D per week	4	10	27·7	27·7	66·7	69·9	86·7	92·3	20·0	22·4

The differences between the increases in weight of the different groups of rats cannot be regarded as significant. It is evident therefore (*a*) that 8 International units of vitamin D per week were sufficient for rats of this colony, and (*b*) that an excess of vitamin D has no harmful effect on a vitamin A determination. It is useful to realise this because the determination of vitamin A in cod liver oils always involves the giving of extra vitamin D also, sometimes enough to double the amount of vitamin D that the rat is already receiving.

B. The influence of an excess of vitamin B_1 on the determination of vitamin D.

The increase in weight of rats during a vitamin D determination by the "line test" is often not very great. The reason for this has never been fully investigated. It has generally been assumed

that 76% of yellow maize, freshly ground, provided sufficient vitamin B (complex) for the young growing rat, and that the depressing effect of the diet was due to its high calcium, low phosphorus ratio. However, Bruce and Phillips investigated the effect on a vitamin D determination, of giving rats large doses of vitamin B_1.

The rats were prepared as for an ordinary vitamin D determination by giving them the rachitogenic diet (Steenbock's 2965) for 3 weeks. Then a half of each litter was given 0·15g. or 0·10g. per rat per day of a vitamin B_1 preparation (kindly supplied by Professor Jansen) to make a total of 100 units (1·0g.) during the 10 days' test period. The other half of each litter was given no extra vitamin B_1. Each rat of every litter was given 10 International units of vitamin D at the beginning of the 10 days' test period. At the end of that time the rats were killed and healing assessed in the usual way. There was no significant difference in the average healing of the two groups of rats (Table XII). Thus it was shown that excess of vitamin B_1 has no influence on the determination of vitamin D by the "line test."

TABLE XII

TO SHOW THAT EXCESS OF VITAMIN B_1 DOES NOT INFLUENCE THE RESPONSE TO A DOSE OF VITAMIN D

	No. of rats.	Average healing.	Average increase in weight in 10 days, g.
Rats given 100 units vitamin B_1 and 10 units vitamin D	12	2·68	5·7
Rats given 10 units of vitamin D only	12	2·50	4·4

C. The influence of an excess of vitamin A on the determination of vitamin D.

The amount of vitamin A present in yellow maize which constitutes 76% of Steenbock's rachitogenic diet (2965) is more than sufficient for very rapid growth of the rat. This was shown by Bruce and Phillips, who found that a diet consisting of:

Yellow maize	70%
Wheat gluten	18%
Dried yeast	8%
Salt mixture (Steenbock's 40)	4%

produced a rapid increase in weight in rats which had become steady in weight on the vitamin A-free diet of the laboratory. Seven male rats gained an average of 45·7g. and 7 female rats gained an average of 33·1g. in 3 weeks on the maize diet. The diet had been constituted to be as much like the Steenbock's 2965 diet as possible with the exceptions that (*a*) dried yeast was added to make sure that a possible shortage of vitamin B (complex) should not limit the response, and (*b*) Steenbock's salt mixture was substituted for the calcium carbonate and sodium chloride of the rachitogenic diet, thus removing any possible depressing effect of the high-calcium, low-phosphorus content of the diet and giving the rats one of the best salt mixtures known. The result showed that 70% of yellow maize provided ample amounts of vitamin A, possibly even excessive amounts. More vitamin A than this would certainly be in excess of requirements.

An experiment was therefore carried out on two groups of 11 animals each, each litter of rats being divided equally between the two groups. The rats were prepared as for a vitamin D determination and at the beginning of the test period all of them were given 10 units of vitamin D. During the test period each rat of one group was given a supplement of 40 units of vitamin A as carotene, per day, for the first 9 days. The rats of the other group were given no other supplement. At the end of the test period the examination of the bones was carried out as in the "line" test. The average healing in the rats given the extra vitamin A as carotene was 1·64 and the average healing of the rats given no extra vitamin A was 2·09. This appeared to indicate that the excess carotene had a depressing influence on the effect of vitamin D. A statistical examination of the results indicated that there was only about a 4·5% chance (*i.e.* 1 in 22) of the difference being "due to random sampling" and therefore the difference was probably significant. The test was therefore repeated on 15 pairs of rats, the same supplements being given as before. This time the carotene appeared to enhance the effect of the vitamin D slightly, the average healing in the 15 rats given the extra carotene being 2·02 and that of the rats given no carotene being 1·80. The figures showed that there was a 29% chance of this difference being due to random sampling. Since one test appeared to show that carotene given in addition to a diet already containing abundance of it depressed the action of vitamin D and a second test appeared to show that a similar

addition to the same diet increased the action of vitamin D, and also since there was a very fair chance of both differences being insignificant it must be concluded that excess of vitamin A (as carotene) has no influence on the action of vitamin D.

It therefore appears (*a*) that a partial deficiency of one vitamin in a diet which is used during the determination of another vitamin will influence the result however the test may be arranged : and (*b*) that an excess of any one vitamin in a diet used during the determination of another vitamin will not influence the result Thus one need never be afraid of giving too much of the vitamins of the basal diet ; the danger lies in giving too little.

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PART II

CHAPTER IX

THE STANDARD DEVIATION

As determinations of vitamins become more and more carefully carried out, the more evident does it become that the response of different animals to apparently similar treatment varies greatly, and that this variation in response is uncontrollable. A simple example of this variation is seen in the increase in weight of the young rats of a colony that have been fed on a diet as nearly uniform as it could be made for many years. Not only does the average increase in weight in a given time vary between different litters but the increase in weight of members of the same litter varies. It is not surprising therefore to find the responses of different animals to equal doses of a vitamin varying in a similar way. Doubts arise as to how much faith can be placed in the averages of results which vary as vitamin results vary. The average of one group of results may be twice as great as the average of another group, but the variation in the results within each group may be so great that it is at least questionable, whether the averages are significantly different or not. The following chapters have been written in the hope that they may help students to determine, by recognised statistical methods, to what extent they can believe in their results, assuming, of course, that their technique is as sound as careful scientific training can make it.

1. The Standard Deviation of a Single Observation, σ

When a number of animals have received the same treatment the result of that treatment is usually stated as the average of

the results obtained with the different animals, but the average gives no indication of the range of variation of the different results. A certain calculation can, however, be made from these results, which expresses in a recognised form the range of the variation. The figure obtained by this calculation is called the standard deviation of a single observation. It is usually denoted by the Greek letter σ. It gives the limits on each side of the mean, within which two-thirds of the results (or observations) lie, and outside of which one-third of the results lie. This proportion must not be expected exactly when the number of animals (or observations) is small. The larger the number of animals used the more nearly will the spread of the results approach this proportion. If the standard deviation has been determined from a sufficiently large number of animals it is allowable to predict that if the same experiment is performed on a further large number of animals, then the same proportion of the results from the second batch of animals will lie between the same limits. From this figure for the standard deviation, the proportion of results lying within (or outside) any other limits can be calculated. For example, half the results or observations will lie within the mean plus or minus two-thirds of σ, *i.e.* within $M \pm \frac{2}{3}\sigma$, and the other half will lie outside these limits. Similarly, 95·5% of the observations will lie within $M \pm 2\sigma$ and 4·5% (one-twenty-second) outside these limits, and 99% of the results will lie within $M \pm 2{\cdot}576\sigma$.

It follows then, that when the standard deviation of a certain type of observation has been determined from a large number of these observations, that there is one chance in three that the next single observation will lie outside the mean plus or minus the standard deviation. If there is one chance in three that the next single observation will lie outside $M \pm \sigma$, then there is one chance in six that it will be greater than $M + \sigma$ and one chance in six that it will be less than $M - \sigma$.

Similarly, there is a one-in-two chance that any single observation will lie outside $M \pm \frac{2}{3}\sigma$, and a one-in-twenty-two chance (approximately 4·5%) that it will lie outside $M \pm 2\sigma$. Tables have been constructed denoting the probability of a result lying outside any other limits (Fisher, *Statistical Methods for Research Workers*, 9th Edition, 1944).

Example.—One hundred female rats of a stock colony made the increases in weight shown in column 2 of Table XIII during

the period between the day they were 5 weeks old and the day they were 10 weeks old. They were the first 100 rats put to breeding, June 1930 to March 1931, from the second generation of a colony fed on a particular diet in the writer's laboratory. They were not selected in any way from the mass of rats put to breeding. The average (or mean) increase in weight during this 5 weeks' period was 56·6g. To find the standard deviation, σ, of a single observation from these 100 results,

(1) write down all the observations in a single column (column 2 of Table XIII) and calculate the average of the 100 observations ($=$M or $\bar{x}$) ;

(2) subtract the average value from each observation and put down the deviations from the mean $(x-\bar{x})$ in two columns, positive and negative; check the arithmetic by summing the positive deviations and the negative deviations separately and see that these totals agree (column 3) ;

(3) square each deviation and put down the squares, $(x-\bar{x})^2$, in the next column (column 4). These values are all positive ;

(4) add up the squares of the deviations $=\Sigma(x-\bar{x})^2$;

(5) divide the sum of the squares by the figure which is less by one than the number of observations $(n-1)$. When the number of animals is small $n-1$ is used as the divisor; when the number is large n is used although then it is not very different from $n-1$. For all animal experiments use $n-1$. This result gives the "variance" $=\dfrac{\Sigma(x-\bar{x})^2}{n-1}$;

(6) take the square root of the variance. This result is the standard deviation, σ, of a single observation. Some workers use the letter, s, to denote the standard deviation calculated from a limited number of observations, and reserve σ for the true value which would have had to be calculated from an unlimited number of observations.

Then $$v=\frac{\Sigma(x-\bar{x})^2}{n-1}=\frac{17{,}476\cdot00}{100-1}=\frac{17{,}476\cdot00}{99}=176\cdot52$$

and $$\sigma=\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}=\sqrt{176\cdot52}=\pm13\cdot3$$

TABLE XIII

CALCULATION OF THE STANDARD DEVIATION OF A SINGLE OBSERVATION ON THE INCREASE IN WEIGHT OF FEMALE RATS FROM THE END OF THE 5TH WEEK TO THE END OF THE 10TH WEEK OF LIFE.

Rat.	Increase in weight from 5th to 10th week of life, x.	Difference between increase in weight of each rat and the average increase in weight ($\bar{x}$) $x-\bar{x}$. + difference.	− difference.	Square of difference (or deviation) from mean, $(x-\bar{x})^2$.
1	55		1·6	2·56
2	58	1·4		1·96
3	56		0·6	0·36
4	56		0·6	0·36
5	61	4·4		19·36
6	60	3·4		11·56
7	25		31·6	998·56
8	48		8·6	73·96
9	56		0·6	0·36
10	54		2·6	6·76
11	51		5·6	31·36
12	59	2·4		5·76
13	43		13·6	184·96
14	60	3·4		11·56
15	49		7·6	57·76
16	48		8·6	73·96
17	48		8·6	73·96
18	54		2·6	6·76
19	52		4·6	21·16
20	21		35·6	1,267·36
21	33		23·6	556·96
22	33		23·6	556·96
23	63	6·4		40·96
24	70	13·4		179·56
25	64	7·4		54·76
26	70	13·4		179·56
27	61	4·4		19·36
28	47		9·6	92·16
29	50		6·6	43·56
30	61	4·4		19·36
31	59	2·4		5·76
32	59	2·4		5·76
33	48		8·6	73·96
34	74	17·4		302·76
35	58	1·4		1·96
36	46		10·6	112·36
37	51		5·6	31·36
38	48		8·6	73·96
39	58	1·4		1·96
40	35		21·6	466·56
41	69	12·4		153·76
42	72	15·4		237·16
43	47		9·6	92·16
44	42		14·6	213·16
45	39		17·6	309·76
46	35		21·6	466·56
47	36		20·6	424·36
48	62	5·4		29·16
49	76	19·4		376·36
50	72	15·4		237·16

TABLE XIII—*continued*

Rat.	Increase in weight from 5th to 10th week of life, x.	Difference between increase in weight of each rat and the average increase in weight ($\bar{x}$). $x-\bar{x}$. + difference.	− difference.	Square of difference (or deviation) from mean, $(x-\bar{x})^2$.
51	74	17·4		302·76
52	62	5·4		29·16
53	51		5·6	31·36
54	65	8·4		70·56
55	67	10·4		108·16
56	58	1·4		1·96
57	25		31·6	998·56
58	62	5·4		29·16
59	47		9·6	92·16
60	41		15·6	243·36
61	29		27·6	761·76
62	55		1·6	2·56
63	33		23·6	556.96
64	54		2·6	6·76
65	72	15·4		237·16
66	69	12·4		153·76
67	53		3·6	12·96
68	65	8·4		70·56
69	64	7·4		54·76
70	63	6·4		40·96
71	68	11·4		129·96
72	77	20·4		416·16
73	59	2·4		5·76
74	52		4·6	21·16
75	79	22·4		501·76
76	48		8·6	73·96
77	71	14·4		207·36
78	76	19·4		376·36
79	66	9·4		88·36
80	66	9·4		88·36
81	54		2·6	6·76
82	33		23·6	556·96
83	55		1·6	2·56
84	60	3·4		11·56
85	57	0·4		0·16
86	53		3·6	12·96
87	43		13·6	184·96
88	67	10·4		108·16
89	53		3·6	12·96
90	47		9·6	92·16
91	62	5·4		29·16
92	64	7·4		54·76
93	74	17·4		302·76
94	71	14·4		207·36
95	68	11·4		129·96
96	74	17·4		302·76
97	77	20·4		416·16
98	73	16·4		268·96
99	69	12·4		153·76
100	83	26·4		696·96
Total	5,660	+528·2	−528·2	17,476·00
Mean	$56·6=\bar{x}$	$\underbrace{\qquad}_{=0}$		$=\Sigma(x-\bar{x})^2$

To test the value 13·3 of σ for the above set of observations: According to theory

(a) one-half of the observations should lie outside the values $M+\frac{2}{3}\sigma=65{\cdot}5$ and $M-\frac{2}{3}\sigma=47{\cdot}7$;

(b) one-third of the observations should lie outside the values $M+\sigma=69{\cdot}9$ and $M-\sigma=43{\cdot}3$;

(c) one-twenty-second of the observations should lie outside the values $M+2\sigma=83{\cdot}2$ and $M-2\sigma=30{\cdot}0$.

Count the number of observations that actually lie outside these limits and compare the numbers found with the numbers given by the theory.

	Number of observations outside the limits.	
	(a) According to theory.	(b) Actually found.
$M\pm\frac{2}{3}\sigma$	50·0	48
$M\pm\sigma$	33·3	32
$M\pm2\sigma$	4·5	4

Considering the smallness of the number (n) of observations used in this example, the agreement between found and calculated results may be said to be very good. Such good agreement must not be expected in every group of a hundred animals examined.

The standard deviation, s, may also be calculated as

$$\sqrt{\frac{\Sigma x^2-n\bar{x}^2}{n-1}}$$

e.g. Suppose the following amounts of healing had been brought about in one group of rats in a "line test" for vitamin D. s could be determined by either method.

Method 1.				Method 2.
Healing.	$x-\bar{x}$ +	$x-\bar{x}$ −	$(x-\bar{x})^2$	x^2
1·5	0·3		0·09	2·25
1·0		0·2	0·04	1·00
0·5		0·7	0·49	0·25
2·0	0·8		0·64	4·00
1·5	0·3		0·09	2·25
1·5	0·3		0·09	2·25
0·5		0·7	0·49	0·25
1·0		0·2	0·04	1·00
1·0		0·2	0·04	1·00
1·5	0·3		0·09	2·25
10) 12·0	+2·0	−2·0	2·10	16·50
$\bar{x}=$ 1·2				

Whence $s=\sqrt{\frac{2{\cdot}10}{9}}$
$=\sqrt{0{\cdot}2\dot{3}}$
$=0{\cdot}483$

Whence $s=\sqrt{\frac{16{\cdot}50-10\times(1{\cdot}2)^2}{9}}$
$=\sqrt{0{\cdot}2\dot{3}}$
$=0{\cdot}483$

When the observations are small numbers, *i.e.* with few figures, the second method is often simpler, and especially is it simpler when the average contains more figures than the observations themselves.

The basis of the calculation

H. Poincaré wrote in the Preface to his *Thermodynamique* (Paris, 1892), "Un physicien éminent me disait un jour à propos de la loi des erreurs : 'Tout le monde y croit fermement parceque les mathématiciens s'imaginent que c'est un fait d'observation, et les observateurs que c'est un théorème de mathématiques.' "

Certainly when tested on large enough groups of animals which have been treated as much alike as possible, the observations have demonstrated the validity of the theory over and over again.

The distribution or scatter of observations is often depicted

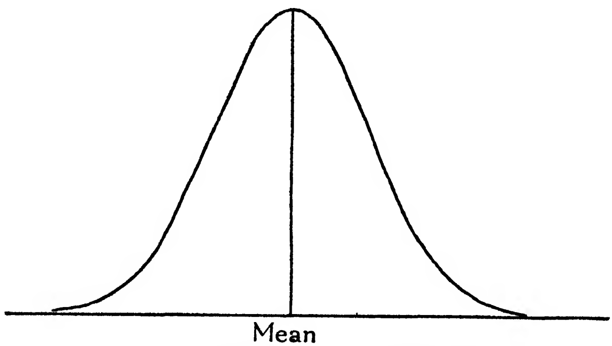

FIG. 34.—Normal curve of distribution.

graphically. If the number of observations is large enough, a smooth curve is obtained. Suppose that, instead of the hundred rats in the example given above, there had been a thousand or ten thousand or, preferably, a million. If the number of rats which showed each increase in weight were plotted against those increases in weight, a curve such as that depicted in Fig. 34 would be obtained, provided the observations were indeed normally distributed, *i.e.* provided that no disturbing factor had been introduced which had made it not merely one set of observations but more.

In Fig. 34 the mean, M, of say, a million (or many millions) observations has been placed as an abscissa at a convenient distance from 0. The number of observations exactly equal to M (*i.e.* the number of rats whose increases in weight were each

equal to M) has been plotted against M. The number of observations exactly equal to M+1 and M−1 have been plotted against those respective points, the number equal to M+2 and M−2 against those, and so on until the point is reached theoretically beyond which no observations on the whole million were obtained. Then if all the observations of the values M, M±1, M±2, M±3, . . . $M \pm \frac{2}{3}\sigma$ (whatever σ has been found by calculation to be) are added together, they would amount to half a million, and if all the observations outside the limits $M \pm \frac{2}{3}\sigma$ were added together they would amount to half a million. Similarly, all the observavations lying outside $M \pm \sigma$ would add up to one-third of a million. All the observations lying outside $M \pm 2\sigma$ would add up to about 4·5 % of the million. All the observations lying outside $M \pm 2{\cdot}576\sigma$ would add up to 1·0 % of the million. The limits outside of which any other proportion of observations lie may be found from the appropriate table ("Table of x" in Fisher's *Statistical Methods for Research Workers*, 9th Edition, 1944).

2. Test of Normalcy of the Distribution of Results

Sooner or later, workers on the determination of the vitamins must discover whether the variations in the results of their tests are indeed normally distributed; that is, whether they form a "normal" curve when enough results are available and they are plotted as in Fig. 34. This can be done by calculations much more satisfactorily than by plotting, for it is seldom that enough results are collected to form a really smooth curve. Two calculations are made:

(*a*) $\mu_3 = \frac{\Sigma(x-\bar{x})^3}{n}$, called the "third moment."

(*b*) $\mu_4 = \frac{\Sigma(x-\bar{x})^4}{n}$, called the "fourth moment."

The values of μ_3 and μ_4 are calculated in a way similar to the calculation of $\mu_2 = \frac{\Sigma(x-\bar{x})^2}{n}$ which is called the "second moment."

Then, the more nearly the value of β_1 which $= \frac{\mu_3^2}{\mu_2^3}$ approximates to the value 0, the more nearly is the curve symmetrical. The more nearly the value of β_2 which $= \frac{\mu_4}{\mu_2^2}$ approximates to the value of 3·0, the more nearly does the curve have the right height in proportion to its width.

Example.—In the writer's laboratory the variation in response to doses of vitamin A has been worked out by this method and found to be normal.

The number (n) of male rats in the group of tests used for this calculation was 922. Σd^2, Σd^3 and Σd^4 were calculated in the usual way.

Then $$\mu_2=\frac{\Sigma d^2}{n}=\frac{+117{,}063}{922}=126{\cdot}97$$

$$\mu_3=\frac{\Sigma d^3}{n}=\frac{-175{,}283}{922}=-190{\cdot}11$$

$$\mu_4=\frac{\Sigma d^4}{n}=\frac{+41{,}492{,}207}{922}=45{,}002{\cdot}39$$

and $$\beta_1=\frac{\mu_3{}^2}{\mu_2{}^3}=\frac{(-190{\cdot}11)^2}{126{\cdot}97^3}=0{\cdot}018$$

therefore the curve is symmetrical.

$$\beta_2=\frac{\mu_4}{\mu_2{}^2}=\frac{45{,}002{\cdot}39}{126{\cdot}97^2}=2{\cdot}79$$

therefore the curve is slightly flat.

The number of female rats in the group used for this calculation was 1,174.

Then $$\mu_2=\frac{+92{,}965}{1{,}174}=79{\cdot}19$$

$$\mu_3=\frac{-277}{1{,}174}=-0{\cdot}24$$

$$\mu_4=\frac{+26{,}213{,}467}{1{,}174}=22{,}319{\cdot}82$$

and $$\beta_1=\frac{\mu_3{}^2}{\mu_2{}^3}=\frac{(-0{\cdot}24)^2}{79{\cdot}19^3}=0{\cdot}000000115$$

therefore the curve is symmetrical.

$$\beta_2=\frac{\mu_4}{\mu_2{}^2}=\frac{22{,}319{\cdot}82}{79{\cdot}19^2}=3{\cdot}5$$

therefore the curve is a little peaked.

Therefore the variation in response to doses of vitamin A may be said to be normal, or the deviations from the mean response may be said to be normally distributed.

3. The Standard Error of the Mean (ϵ) (sometimes called the Standard Deviation of the Mean)

Just as a figure can be obtained to express the probability that a single observation will lie outside certain limits on each side of the mean, so a figure can be obtained to express the probability that an average result from any given number of animals will lie outside certain limits on each side of the mean result obtained from all the animals investigated. That is, if several hundred animals have been experimented upon, giving a value for σ which may be regarded as approaching the true value for σ, it is possible to calculate the chance that the average result of a small group of, say, 10 animals will lie outside certain limits on each side of the true mean M. The calculation is made from the standard deviation of a single observation by dividing it by the square root of the number of animals used, *i.e.* $\epsilon = \frac{\sigma}{\sqrt{n}}$.

Then, just as

50%	of the single observations	lie	outside	$M \pm \frac{2}{3}\sigma$
33·3%	,,	,,	,,	$M \pm \sigma$
4·5%	,,	,,	,,	$M \pm 2\sigma$

So, if all the single observations are divided without selection into groups of a certain number of animals,

50%	of the averages of the groups	lie	outside	$M \pm \frac{2}{3}\epsilon$
33·3%	,,	,,	,,	$M \pm \epsilon$
4·5%	,,	,,	,,	$M \pm 2\epsilon$

Example.—The mean increase in weight of the 100 rats from which the calculation on p. 173 was made was 56·6g., which may or may not have been near the true mean for the colony. The standard deviation of a single observation of the increase in weight was found to be $\pm 13{\cdot}3$g. This means that one-third of the 100 rats made increases in weight outside the limits $M \pm \sigma$, *i.e.* greater than 69·9g. or less than 43·3g.

Now the standard error, ϵ, of the average result of a group of 10 of these rats is found by dividing the standard deviation by the square root of the number of animals in the group—

i.e. $$\epsilon = \frac{\sigma}{\sqrt{n}} = \pm \frac{13{\cdot}3}{\sqrt{10}} = \pm \frac{13{\cdot}3}{3{\cdot}16} = \pm 4{\cdot}2\text{g.}$$

This means that, of all the groups of 10 animals,

$\frac{1}{2}$ of the groups should have a mean lying outside $56{\cdot}6 \pm \frac{2}{3} \times 4{\cdot}2$g.

$\frac{1}{3}$ of the groups should have a mean lying outside $56{\cdot}6 \pm 4{\cdot}2$g.

$\frac{1}{22}$ of the groups should have a mean lying outside $56{\cdot}6 \pm 2 \times 4{\cdot}2$g.

$\frac{1}{100}$ of the groups should have a mean lying outside $56{\cdot}6 \pm 2{\cdot}576 \times 4{\cdot}2$g.

This last corresponds to $P = 0{\cdot}99$, the limits of error adopted for specified numbers of animals in the 1936 Addendum to the British Pharmacopœia, 1932.

It is suggested that the student should now divide the 100 figures given in column 2 of Table XIII into ten groups of 10 rats each, taking them in sequence exactly as they occur in the table ; then find the average of each group and see what proportions of the ten groups lie outside the expected limits for the groups. He should not expect a very good agreement between found and calculated proportions as he has only ten of these groups with which to work.

The above results may be stated in another way. When once the standard deviation of a single observation and from it the standard error of the mean of a group of 10 animals have been found, the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm \frac{2}{3}\epsilon$ is 1 in 2. Similarly, the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm \epsilon$ is 1 in 3, and the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm 2\epsilon$ is 1 in 22. Also the chance that a similar test on a group of 10 animals will give an average result lying outside $M \pm 2{\cdot}576\epsilon$ is 1 in 100.

It should be realised at this stage that since $\epsilon = \frac{\sigma}{\sqrt{n}}$ the accuracy of an average result is inversely proportional to the square root of the number of animals used, *e.g.* to halve the inaccuracy of an average result, four times the number of animals must be used ; 36 animals are required to reduce to one-half the inaccuracy of an average result in which 9 animals were used, and using 100 animals is only twice as good as using 25 animals. This should be considered when a decision has to be made as to how many animals should be used in any experiment.

The "probable error," a term frequently used in biological estimations, is the value of $\frac{2}{3} . \epsilon$, *i.e.* $\frac{2}{3} . \frac{\sigma}{\sqrt{n}}$. It may be used in a general sense for a particular type of determination whose standard deviation has been determined from experiments on a very large number of animals; and then the "probable error" must be accompanied by a statement of the number of animals used, *e.g.* "the probable error of a test when a group of 10 animals is used, is . . ." The term may also be used for expressing the error of an experiment which has been performed only once on a group of, say, 5 animals, when the probable error of the mean of the five results would be found first by determining σ for that group of 5 animals, then dividing it by $\sqrt{5}$ and then multiplying it by $\frac{2}{3}$, *i.e.* the probable error of the mean was $\frac{2}{3} . \frac{\sigma}{\sqrt{5}}\left(=\frac{2}{3}\sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}} \times \frac{1}{\sqrt{n}}=\frac{2}{3}\sqrt{\frac{\Sigma(x-\bar{x})^2}{n(n-1)}}\right)$.

4. The Difference between Two Means

Workers often find such a divergency in the individual results in two groups of animals used in a comparison that they seriously doubt whether the apparent difference between the averages of the two groups really means anything or not, *i.e.* whether the difference between the two means is significant or not. A calculation can be made to determine this.

The standard deviation of the results given by each group of animals is determined, and from this the standard error of each group. These are substituted in the formula $t=\frac{\bar{x}_1-\bar{x}_2}{\sqrt{\epsilon_1^2+\epsilon_2^2}}$ in which $\bar{x}_1$ is the average result of one group and ϵ_1 is its standard error, $\bar{x}_2$ is the average result from the other group and ϵ_2 is its standard error. If on working out the value obtained by these substitutions the result is found to be greater than 3, the difference between $\bar{x}_1$ and $\bar{x}_2$ may be accepted as significant, *i.e.* there is a good probability that a real difference exists. Some workers are indeed satisfied if they find the value to be greater than 2. This is a matter of temperament. It really means that if the result is greater than 3 there is a stronger chance of the difference being significant than if the result is greater than 2 and less than 3.

The statement written above may be expressed thus: If the difference between the means is more than three times the

square root of the sum of the squares of the standard errors of the groups used, then this difference is significant.

To determine more exactly the probability that the difference between $\bar{x}_1$ and $\bar{x}_2$ is significant, consult the table of t in Fisher's *Statistical Methods for Research Workers*. Suppose the two groups had consisted of 8 and 6 animals respectively. Under the column headed n in the table look for the figure 12 (*i.e.* $8-1+6-1$). On the horizontal line for $n=12$, look for the nearest figure to your value of t. If this is, say, 2·531, it will be found between the two values 2·179 and 2·681 which come in the columns headed 0·05 and 0·02 respectively. Therefore there is less than a 0·05 (or 5%) chance that the difference between $\bar{x}_1$ and $\bar{x}_2$ is due to chance; that is the difference may be considered significant.

Suppose, however, that the groups had consisted of only 4 animals each. Here $n=6$ (*i.e.* $4-1+4-1$) and in this horizontal line in the table, the value 2·531 falls between the columns headed 0·1 and 0·05. This indicates that the difference is barely significant, as no probability greater than $P=0·05$ can really be accepted, but it would be well worth while to repeat the experiment.

5. References

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CHAPTER X

FACTORS INFLUENCING THE ACCURACY OF VITAMIN DETERMINATIONS

1. The Standard Deviation of the Observations, s, and of the Mean, e.
2. The Slope of the Curve of Response, b, (a) a logarithmic curve, (b) a rectilinear curve.
3. The Logarithmic Variance of the Result, λ or $\left(\frac{s}{b}\right)^2$.
4. The Value of $\bar{y}_1 - \bar{y}_2$.
5. The Significance of the Slope of the Curve of Response.
6. The Error of the Slope of the Average Curve of Response.
7. References.

1. The Standard Deviation of the Observations, s, and of the Mean, e.

SINCE all determinations of vitamins are made on a very limited number of animals, the standard deviation will be represented by the letter s, instead of the Greek letter σ which represents the true standard deviation determined from an unlimited number of animals.

It is obvious that the smaller the value of s in any experiment, the smaller is the inaccuracy or error of that experiment. It is equally obvious that the larger the number of animals in a group the smaller will be the error, e. Moreover, the value of s varies from one experiment to another, in a quite uncontrollable way. If an average value of s is required (for reference or comparison), it is not obtained by averaging the several values from the various experiments, but by averaging the variances weighted according to the number of rats in each group or preferably to the number of rats minus one ($n-1$) in each group, *e.g.*

Group.	No. of rats.	s.	s^2.	$(n-1)s^2 = \Sigma d^2 = \Sigma(x-\bar{x})^2$
1	10	16·44	270·40	2433·60
2	8	16·97	288·00	2016·00
3	6	7·59	57·66	288·30
4	5	9·84	96·80	387·20
5	9	22·50	506·25	4050·00
				9175·10

$$\text{Average variance} = \frac{9175 \cdot 10}{9+7+5+4+8} = 278 \cdot 033$$

and the average $s=\sqrt{278\cdot033}=16\cdot67$ which is different from the average of the 5 values of s (14·67) and from the average value of s weighted according to the number of animals in each group (15·72), and from the average value of s weighted according to the number of animals minus one in each group (16·56).

Coward showed in 1932, and much more conclusively in 1942, that the standard deviation of the increase in weight of rats in a vitamin A test did not vary with the increase in weight during the test. Thus an average value for s may be obtained from experiments in which the average increases in weight vary widely, provided the curve of response is logarithmic.

2. The Slope of the Curve of Response, *b*

(*a*) *A logarithmic curve.*—It has been explained in Part I that the potencies of two doses of vitamin A are not directly proportional to the average increases in weight which are brought about in the rats by those doses. The relationship between dose and effect is a curvilinear one. Therefore the probable error of the result of giving a particular dose of vitamin A to 10 rats cannot be obtained by determining the ratio between $M-\frac{2}{3}\epsilon$, M, and $M+\frac{2}{3}\epsilon$, or by stating $M-\frac{2}{3}\epsilon$ and $M+\frac{2}{3}\epsilon$ as a percentage of M. It is necessary to find the abscissæ corresponding to M, $M+\frac{2}{3}\epsilon$ and $M-\frac{2}{3}\epsilon$ from the curve relating response to dose given and then find the ratio of these abscissæ, or express the abscissæ corresponding to $M\pm\frac{2}{3}\epsilon$ as percentages of the abscissa corresponding to M. The abscissæ may be determined directly from the curve drawn on graph paper or by calculation from the equation representing the curve.

Example.—Suppose the average increase in weight of 10 male rats given a dose of vitamin A daily for 5 weeks was 20g., and the standard deviation of the increase in weight was ±15g. Then the standard error using 10 rats would be $\epsilon=\pm\dfrac{15}{\sqrt{10}}=\pm4\cdot75$g., and the probable error would be $\frac{2}{3}\epsilon$ which is ±3·17g. Suppose the curve of response for tests of this kind was $y=10+50\log. x$. Then the abscissæ of this curve corresponding to $y=M=20$g., $y=M+\frac{2}{3}\epsilon=23\cdot17$g. and $y=M-\frac{2}{3}\epsilon=16\cdot83$g. are respectively 1·58, 1·83 and 1·36, from which the percentage error is calculated to be +16% or −13% (Fig. 35 (a)).

Suppose, however, that the curve of response was represented by the equation $y=10+30 \log. x$. The calculation made from the same results, but with the use of this less steep curve, shows the abscissæ corresponding to M, M$+\frac{2}{3}\epsilon$, M$-\frac{2}{3}\epsilon$ to be 2·14, 2·75 and 1·69 respectively, and that the probable error of the result is $+28$% or -21% (Fig. 35 (b)).

It is evident that the steeper the curve of response relating effect to dose given, the more accurate (or less inaccurate) is the result. Thus two factors contribute towards the estimate of the

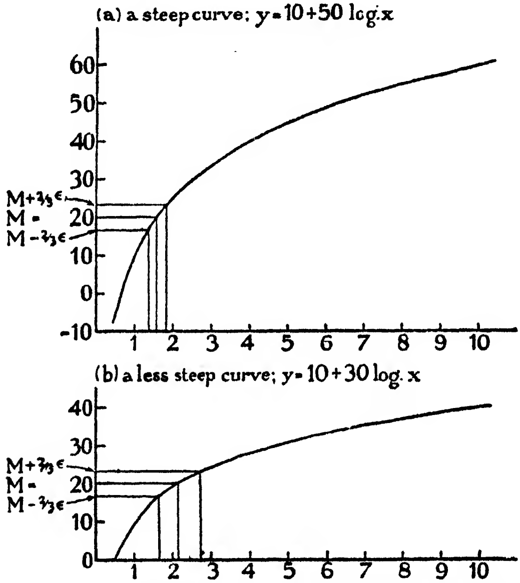

FIG. 35.—To find the probable error of a result from $\frac{2}{3}\epsilon$ and the curve of response.

accuracy of a biological test: (*a*) the standard error (which combines the standard deviation and the number of animals used), and (*b*) the steepness of the curve of response. The greater the standard deviation the greater the error, the greater the number of animals used the smaller the error, and the steeper the curve of response the smaller is the error.

Moreover, when the standard deviation does not vary with the result (*e.g.* average increase in weight) and the curve of response is logarithmic, the same percentage error will be obtained whether the result is high or low. Will the student

convince himself of this, if necessary, by working it out for two or three increases in weight and one curve of response, *e.g.* $y=10+50 \log. x$.

(*b*) *A rectilinear curve.*—When, however, the curve of response is linear, *i.e.* the response varies simply with the dose, then the percentage error calculated from a particular standard deviation will vary inversely with the average response. An example of the kind of curve is seen in the response of guinea-pigs to doses of vitamin C as determined by the Key and Elphick method (1931). It is possible, of course, that some animal reaction might give a larger standard deviation with a larger dose and a linear curve of response such that the percentage error would be the same for all doses and for all responses, but the writer does not know such a reaction. (See also Finney, 1945.)

3. The Logarithmic Variance of the Result, λ or $\left(\frac{s}{b}\right)^2$

The standard deviation divided by the slope of the curve of response gives a measure of the accuracy of the result called the standard deviation of the logarithm (to the base 10) of the result. It is designated by the Greek letter λ and the logarithmic variance by λ^2. Obviously, the greater the standard deviation the greater the logarithmic variance; and the greater the slope of the curve of response the smaller is the logarithmic variance. The logarithmic variance thus combines two factors which influence the accuracy of the result.

The slope of the curve of response is given by the coefficient of $\log. x$ in the equation representing the curve of response. This figure is the tangent of the angle which the curve makes with the X axis when the increases in weight are plotted against the logs. of the doses instead of against the doses themselves. Thus the slope of the curve $y=11{\cdot}3+50{\cdot}3x$ is 50·3 and that of the curve $y=12{\cdot}4+27{\cdot}4x$ is 27·4. The values of λ for male and female rats respectively from figures obtained in vitamin A determinations in the writer's laboratory (Coward, 1932) are found thus:

	s.	Slope of curve of response.	$\lambda=\frac{s}{\text{slope}}$.
Male rats	14·72	50·3	0·29
Female rats	11·02	27·4	0·40

The ratio of the values of λ obtained from male and female rats is thus 0·29 : 0·40, which is about 1 : $\sqrt{2}$, and since the standard error of a test is inversely proportional to the square root of the number of animals used, it is evident that twice as many female rats as male rats must be used in a test to get the same degree of accuracy.

4. The Value of $\bar{y}_1 - \bar{y}_2$

Still another factor influences the accuracy of a vitamin determination. Nowadays, a test is generally arranged to compare two doses of the Standard in the ratio of 2 : 1 or 3 : 1 with two doses of the substance being tested, in the same ratio. Sometimes the two curves obtained by plotting the average responses against the abscissæ 0 and 0·3010 (log. 2) or 0 and 0·4771 (log. 3) lie almost on top of one another, with more or less the same slope, but sometimes, when the doses have been less fortunately chosen, one curve may be much above the other. The average value of $\bar{y}_1$ from all the rats on both doses of the Standard is compared with the average value of $\bar{y}_2$ from all the rats on both doses of the substance being tested. The more nearly $\bar{y}_1 = \bar{y}_2$, the greater the accuracy of the test (see the next chapter).

5. The Significance of the Slope of the Curve of Response

The variance of the response of the animals to one dose and to double or treble of that dose may be so great that the slope of the curve relating average response to the log. of the dose given is not significant, *i.e.* the average response to the larger dose is not significantly larger than that to the lower dose. Such a curve of response is obviously useless.

The slope of a curve is significant if $b^2 > t^2 s_b{}^2$ where b is the slope of the curve, t is the factor from the t table corresponding to the probability desired and the number of rats minus 2 (if 2 doses have been used) with which the table is entered, and

$$s_b{}^2 = \text{the variance of the slope}$$
$$= \frac{s^2 \text{ (the variance of the observations)}}{S(x-\bar{x})^2}.$$

Some writers use B for $s_b{}^2$, *e.g.* Irwin (1943), and put $B = \frac{s^2}{S\{w(x-\bar{x})^2\}}$ which is only the same as the formula written above with w inserted to indicate the number of observations.

Example.—Suppose the increases in weight recorded in Col. 1 below had been gained by rats which had been given 1 and 2 I.U. respectively of vitamin A. Then:

	Increase.	Difference from mean. +	Difference from mean. −	Difference².	
1·0 I.U.	31	13		169	
	22	4		16	
	16		2	4	
	19	1		1	
	8		10	100	
	7		11	121	
	15		3	9	
	20	2		4	
	10		8	64	
	32	14		196	
	10 | 180	+34	−34	684	
	$\bar{x}_1 =$ 18·0				$s_1^2 = \frac{684}{10-1} = 76{\cdot}0$
2·0 I.U.	29	5		25	
	46	22		484	
	32	8		64	
	16		8	64	
	23		1	1	
	19		5	25	
	11		13	169	
	24	0	0	0	
	23		1	1	
	17		7	49	
	10 | 240	+35	−35	882	
	$\bar{x}_2 =$ 24·0				$s_2^2 = \frac{882}{10-1} = 98{\cdot}0$

The average $s^2 = \frac{684+882}{9+9} = 87$ which is the simple average of 76 and 98, but if there had been different numbers of rats in the two groups, it would not have been.

The slope of the response/log. dose curve from these two doses is given by $b = \frac{24{\cdot}0 - 18{\cdot}0}{\log. 2} = \frac{6{\cdot}0}{0{\cdot}3010} = 19{\cdot}93$.

Now, in the formula $b^2 - t^2 s_b^2$

$b^2 = 19{\cdot}93^2 = 397{\cdot}2049$

$t^2 = 2{\cdot}101^2 = 4{\cdot}414201$ for $P = 0{\cdot}05$ and $n = 18$ (*i.e.* $10-1+10-1$) from the table of t.

Also since $\bar{x}$ is the average of the logs. of the two doses which were in the ratio 2 : 1, each $x - \bar{x} = \frac{1}{2}$ of 0·3010 and there are

10 differences of $+0{\cdot}151$ and 10 differences of $-0{\cdot}151$. Since $(+0{\cdot}151)^2=+0{\cdot}022801$ and $(-0{\cdot}151)^2=+0{\cdot}022801$, $S(x-\bar{x})^2 =20\times 0{\cdot}022801=0{\cdot}45602$.

Then $$s_b{}^2=\frac{s^2}{S(x-\bar{x})^2}=\frac{87}{0{\cdot}456}=190{\cdot}8$$

and $$t^2 s_b{}^2=4{\cdot}414\times 190{\cdot}8=842{\cdot}1912$$

Since $b^2=19{\cdot}93^2=397{\cdot}2049$ is less than $t^2s_b{}^2=842{\cdot}1912$ then the slope of the curve is not significant.

6. The Error of the Slope of the Average Curve of Response

The standard deviation of the average curve of response obtained from two doses of the Standard and two of the substance being tested is found from the equation:

$$s_b{}^2=\frac{s^2}{S(x_1-\bar{x}_1)^2+S(x_2-\bar{x}_2)^2}$$

where s^2 is the average variance found from all four groups, $S(x_1-\bar{x}_1)^2$ is the sum of the squares of all the differences between the doses of Standard and their mean and $S(x_2-\bar{x}_2)^2$ is the sum of the squares of all the differences between the doses of the substance under test and their mean.

e.g. A simple case—suppose two groups of 8 rats each had been given doses of 1 and 2 units respectively of vitamin B_1 in a "curative" test and two other groups of 7 rats each had been given doses of 1 and 2 grammes of a food substance. The curve of response to doses of vitamin B_1 is logarithmic. Then the logs. of the doses for either Standard or food substance are 0 and $0{\cdot}3010$, and $\bar{x}_1=\dfrac{0{\cdot}3010}{2}=0{\cdot}150$ and $\bar{x}_2=\dfrac{0{\cdot}3010}{2}=0{\cdot}150$ and $(x_1-\bar{x}_1)^2=(0{\cdot}150)^2$ whether x_1 stands for the log. of the higher $(0{\cdot}3010)$ or lower dose (0) and $(x_2-\bar{x}_2)^2$ also $=(0{\cdot}150)^2$.

Then, since there were 16 rats on the Standard and 14 rats on the food substance, and using s for the limited numbers of animals rather than σ for the unlimited number,

$$s_b{}^2=\frac{\text{Variance of the responses, say } 25{\cdot}0}{16\times(0{\cdot}150)^2+14\times(0{\cdot}150)^2}$$
$$=\frac{25{\cdot}0}{30\times 0{\cdot}0225}$$
$$=37{\cdot}04$$

and s_b will therefore be $6{\cdot}086$.

Taking into account the distance between the two curves made parallel with average slope through the "mid-points" of the two actual curves, the whole error of the average slope is determined by the formula

$$\text{Error} = \frac{(\bar{y}_1 - \bar{y}_2)^2 s_b^2}{b^4}$$

where $\bar{y}_1$ is the average of all the responses to both doses of standard and $\bar{y}_2$ is the average of all the responses to both doses of the food substance.

7. References

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CHAPTER XI

THE LIMITS OF ERROR OF A DETERMINATION OF A VITAMIN

1. Approximate Limits of Error of a Determination and the Combination of Two Determinations.
2. Exact Fiducial Limits of a Determination.
   (*a*) in which the response is graded to the log. of the dose given.
   (*b*) in which the response is an "all-or-none" reaction.
3. Analysis of Variance.
4. References.

In a lecture to the Agriculture Group of the Society of Chemistry and Industry, Dr. G. H. Bates reports Sir John Russell as having said that "truth might be defined as something which had a twenty to one chance of being right." When the limits or error of a test are said to be, say, 79 to 126% for $P=0{\cdot}95$, it means that there is one chance in twenty of the result obtained being less than 79 or greater than 126% of the true value. The same experiment would have limits of error of 74 to 135% for $P=0{\cdot}99$, *i.e.* there would be one chance in a hundred of the value obtained being either less than 74 or greater than 135% of the true value. In view of possible legal proceedings the Pharmacopœia Commission adopted the $P=0{\cdot}99$ probability as amounting to almost certainty.

Until a very few years ago the limits of error were calculated by a method which has recently been shown to give only approximate figures. These are called approximate limits in contrast to the true fiducial limits obtained by the methods more recently devised. The calculation of the approximate limits is very much simpler than that of the true fiducial limits. It still has a use of some importance in finding the limits of error of a combination of several determinations of the same substance by the same method. (Table XIV.)

Bliss (1945) uses the term "limits of confidence" for the term "fiducial limits" of British workers.

1. Approximate Limits of Error of a Determination and the Combination of Two Determinations

Approximate limits of error may be used if the slope is greater than 7·75 times its standard error. The variance (S^2) of the log. of the result obtained by the procedure described in Chapters IV and VII is calculated thus :

Suppose the two parallel straight lines fitted to the two curves for standard and test substance respectively, through their "mid-points" are represented by the equations :

$$y_1=\bar{y}_1+b(x_1-\bar{x}_1) \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

and

$$y_2=\bar{y}_2+b(x_2-\bar{x}_2) \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

then we can get the horizontal distance between these lines at any point y which is the same for both of them, *i.e.* $y_1=y_2$ or $y_1-y_2=0$.

If equation (1) is subtracted from equation (2) we get

$$y_1-y_2=\bar{y}_1-\bar{y}_2+b\{x_1-\bar{x}_1-(x_2-\bar{x}_2)\}$$

$$0=\bar{y}_1-\bar{y}_2+b\{x_1-x_2-(\bar{x}_1-\bar{x}_2)\}$$

$$x_1-x_2=\bar{x}_1-\bar{x}_2-\frac{\bar{y}_1-\bar{y}_2}{b}$$

which is the log. of the ratio of the potency of the test substance to that of the standard.

The variance of the log. of the result is given by the formula

$$\frac{s^2}{b^2}\left(\frac{1}{n_1}+\frac{1}{n_2}\right)+\frac{s^2}{S_1(x_1-\bar{x}_1)^2+S_2(x_2-\bar{x}_2)^2}\left\{\frac{(\bar{y}_2-\bar{y}_1)^2}{b^4}\right\}$$

and the standard error of the log. of the result by $\pm$ the square root of this quantity.

Example.—The vitamin A potency of a sample of butter was determined by the "increase in weight" method as described in Chapter IV. Both male and female rats were used. The potency of the butter was calculated from the males and females separately, together with the accuracy of each determination. The results were then combined, weighting according to accuracy and the accuracy of the combined result determined as in Table XIV.

The data of Table XIV were obtained as follows :

Column 2—the results (potency of the butter) as determined from the male and female rats respectively.

TABLE XIV

CALCULATION OF THE LIMITS OF ERROR (P=0·99) FOR A COMBINATION OF TWO RESULTS. THE VITAMIN A POTENCY OF A SAMPLE OF BUTTER DETERMINED ON MALE AND FEMALE RATS BY THE "INCREASE IN WEIGHT" METHOD

1	2	3		4	5	6		7	8	9	10	11	12	13	14	15
Sex of Rats.	Result =R I.U. per g.	No. of rats.		Variance, σ^2.	D/F.	Slope.		Aver. slope, b.	$\sigma^2\left(\frac{1}{n_1}+\frac{1}{n_2}\right)$	$S_1^2=\dfrac{\sigma^2\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}{b^2}$	S_1.	$\dfrac{(\bar{y}_1-\bar{y}_2)^2\sigma_b^2}{b^4}$.	σ_M^2.	$\dfrac{1}{\sigma_M^2}=$W.	M = log.R.	WM.
		St. n_1.	Test n_2.			St.	Test.									
♂	23·81	25	35	202·51	55	45·18	57·99	52·65	13·8864	0·005009	0·070774	0·000092	0·005101	196·04	1·37677	269·901991
♀	28·12	26	41	79·71	62	36·54	35·72	36·04	5·0099	0·003857	0·062105	0·000017	0·003874	258·13	1·44901	374·032951
														454·17		643·934942

(Column 12 is the sum of columns 9 and 11.)

M.	M − $\bar{M}$.	(M − $\bar{M}$)^2.	W.	W(M − $\bar{M}$)^2.
1·37677	0·03612	0·0013047	196·04	0·255773388
1·44901	0·03612	0·0013047	258·13	0·336782211
2)2·82578			454·17	0·592555599 = χ^2
$\bar{M}$ = 1·41289				

$$\frac{\Sigma WM}{\Sigma W} = \frac{643{\cdot}934942}{454{\cdot}17} = 1{\cdot}4178$$
$$= \text{log. } 26{\cdot}170$$

∴ the butter contained 26·2 I.U. vitamin A per g.

$$\sigma_M^2 = \frac{\chi^2}{\Sigma W} = 0{\cdot}001305$$
$$\sigma_M = \pm 0{\cdot}036124$$
$$2{\cdot}617\sigma = \pm 0{\cdot}094536 = \text{log. } 1{\cdot}2432 \text{ or log. } \bar{0}{\cdot}8033$$

∴ the limits of error for P=0·99 are 124·32 to 80·43%

Column 3—the total numbers of rats given doses of standard and butter respectively, not necessarily "litter-mates."

Column 4—the variance of a single response (increase in weight) as determined from the 60 rats by the method described in Chapter X.

Column 5—D/F=degrees of freedom. There were 2 groups of rats on the standard and 3 groups on the butter, therefore the number of degrees of freedom is $25-2+35-3=55$.

Column 6—the slopes of the curves for standard and butter respectively.

Column 7—the average slope weighted according to the total number of rats determining each slope.

Column 8—the error variance of the average increase in weight.

Column 9—the error variance of the dose corresponding to the increase in weight.

Column 10—the standard error (or deviation) of the dose.

Column 11—the variance due to difference between the average responses to standard (2 doses) and test substance (3 doses) respectively. In this σ_b^2 is the variance of the slope of the curve.

Column 12—is the sum of columns 9 and 11 and gives the whole standard error of the log. of the result.

Columns 13, 14 and 15 are self explanatory, put in for averaging the results from male and female rats respectively.

To determine the limits of error of this particular test (on the male rats only) take the square root of 0·005101 which is $\pm 0·071421$ (=S) and the antilog. of this which is 1·1787 or 0·84839. The standard limits of error (*i.e.* a 1 in 3 chance) are therefore 117·87 to 84·84%. The limits of error for P=0·95 (1 in 20 chance) are found by multiplying $\pm 0·0714$ by the value of *t* (found from the Table of *t*) appropriate to the number of degrees of freedom in the test (*i.e.* 55). It may be taken as 2·0. It is certainly less than 2·021 which is the value appropriate to 40 degrees of freedom, and very nearly equal to 2·000 which is the value appropriate to 60 degrees of freedom. Thus $2 \times \pm 0·0714 = \pm 0·1428$ which is the log. of 1·3894 or 0·71978 and the limits of error for P=0·95 are 138·94 to 71·98%. Similarly for P=0·99, multiply $\pm 0·0714$ by the appropriate value of *t*, 2·660 (the value for 60 D/F which is the nearest given in the table). This

gives $\pm 0{\cdot}1899$ which is the log. of $1{\cdot}5485$ or $0{\cdot}64580$, and the limits of error for $P=0{\cdot}99$ are $154{\cdot}85$ to $64{\cdot}58\%$.

The combination of the results from the male and female rats and the limits of error of the final result are shown in Table XIV. The value of t most nearly appropriate to 117 D/F and $P=0{\cdot}99$ given in the Table is $2{\cdot}617$ for 120 D/F.

2. Exact Fiducial Limits of a Determination

The approximate limits of error do not allow for the significance of the slope of the average curve of response.

(*a*) *True fiducial limits of error for a determination in which the response is graded to the log. of the dose* are calculated from the following formula worked out by Irwin (1943):

$$\bar{x}_1-\bar{x}_2-\frac{b(\bar{y}_1-\bar{y}_2)}{b^2-t^2\mathrm{B}}\pm\frac{t}{(b^2-t^2\mathrm{B})}\{\mathrm{A}(b^2-t^2\mathrm{B})+\mathrm{B}(\bar{y}_2-\bar{y}_1)^2\}^{\frac{1}{2}}$$

It gives the actual lower and upper limits for any degree of probability required by using the value of t found in the Table of t in the column headed by the degree of probability required and in the row of the number of degrees of freedom of the experiment. For example, if the limits of error for $P=0{\cdot}95$ are required and 4 groups of 8 rats each had been used, the value of t to be used in this formula is found in the column headed $P=0{\cdot}05$ (the chance that the result will lie *outside* these limits) and in the row where $n=28$ $(=4\times 8-4)$. $t=2{\cdot}048$. For $P=0{\cdot}99$ in the same experiment, $t=2{\cdot}763$ (column for $P=0{\cdot}01$ and row $n=28$).

In Irwin's formula:

$\bar{x}_1$ is the average of the logs. of all the doses of the standard given, weighted according to the number of rats on each dose.

$\bar{x}_2$ is the average of the logs. of all the doses of the test substance given, weighted according to the number of rats on each dose.

b is the average of the slopes from test substance and standard respectively, weighted according to the total numbers of rats on each.

$\bar{y}_1$ is the average response of all the rats on all the doses of the standard.

$\bar{y}_2$ is the average response of all the rats on all the doses of the test substance.

t is described above.

$B = \dfrac{s^2}{S_1\{w(x_1-\bar{x}_1)^2\}+S_2\{w(x_2-\bar{x}_2)^2\}}$ in which s^2 is the estimate of the standard deviation obtained from all the groups as on page 184 or, if litter-mate control has been used, as described in Analysis of Variance on page 205. $S_1\{w(x_1-\bar{x}_1)^2\}$ is the sum of all (w) the squares of the differences between actual doses and the mean dose of standard, and $S_2\{w(x_2-\bar{x}_2)^2\}$ is the corresponding sum for the test substance (w is the number of rats on the standard or on the test substance); when only two doses of each have been given, $x_1-\bar{x}_1$ is then the same for each rat, $+ve$ or $-ve$, and $(x_1-\bar{x}_1)^2$ is always $+ve$.

$A = s^2\left(\dfrac{1}{S_1(w)}+\dfrac{1}{S_2(w)}\right)$ where s has the same value as above, and $S_1(w)$ is the total number of rats given doses of the standard and $S_2(w)$ is the total number of rats given doses of the test substance.

If, in Irwin's formula, $b^2 > t^2B$ (*i.e.* if the slope is significant), then the fiducial limits are real. If $b^2 = t^2B$, the slope is only just significant; the limits for the log. of the potency ratio become $-\alpha$ and $+\alpha$ and for the ratio between test substance and standard the limits are 0 to α, which simply means that no reliance whatever can be placed on the result.

The B.S.I. (1940) formula for determining exact fiducial limits contains the letter C for $\dfrac{b^2}{b^2-t^2B}$ and, by some rearranging, this formula can be put in the form of Irwin's, or *vice versa*.

The fiducial limits of error may also be stated as percentages of the value found. It will be noticed that the ratio of the lower limit to the result is not necessarily (and very rarely is) the same as the ratio of the result to the upper limit, *i.e.* the limits cannot be expressed as the antilog. (log. result $\pm x$).

(*b*) *Fiducial limits of error of a determination in which the response is quantal, i.e. an "all-or-none" reaction,* are calculated from the same formula, but since the response is in units of standard deviation, $S^2=1$, t is the corresponding normal deviate and the weight factor, w, is nz^2/pq (p=the proportion of animals that gave a positive result and $q=1-p$=the proportion which gave a negative result). The value of z^2/pq corresponding to each probit is found from a table given by Bliss (1938) and reproduced

TABLE XV

Calculation of the best curve of response for an "all or none" reaction

Standard

Doses in ratio 1 : 1·5 : 2·25 : 3·375

1	2	3	4	5	6	7	8	9	10	11	12	
x	n	P	Y	M.W.P. $y+Q/Z$ or $y-P/Z$	1/Z	P/Z or Q/Z	y	w	nw	nwx	nwy	
1	10	0·05	3·6	3·061	6·679	0·3339	3·3950	0·3020	3·020	3·020	10·25290	$[wxy]=17\cdot2964$
2	10	0·4	4·5	3·624	2·840	1·1360	4·7600	0·5810	5·810	11·620	27·65560	$[wx^2]=18\cdot8762$
3	12	0·7	5·4	6·336	2·715	0·8145	5·5215	0·6005	7·2060	21·618	39·78793	$b=\dfrac{17\cdot2964}{18\cdot8762}$
4	12	0·9	6·3	6·865	5·835	0·5835	6·2815	0·3359	4·0308	16·1232	25·31947	
									20·0668	)52·3812	103·01590	$=0\cdot9164$
										2·6103	5·1337	

Line with slope 0·9164 through point 2·6103, 5·1337 is $y=0\cdot9164x+2\cdot7416$

			3·6580	3·1039	6·1895	0·3095	3·4134	0·32166	3·2166	3·2166	10·97954	$[wxy]=17\cdot02169$
			4·5744	3·6540	2·7463	1·0985	4·7525	0·59563	5·9563	11·9126	28·30732	$[wx^2]=18\cdot5534$
			5·4908	6·3723	2·8275	0·8482	5·5240	0·58280	6·9936	20·9808	38·63265	$b=\dfrac{17\cdot02169}{18\cdot5534}$
			6·4072	6·9445	6·7540	0·6754	6·2691	0·30163	3·6196	14·4784	22·69163	
									19·7861	)50·5884	100·61114	$=0\cdot9121$
										2·5568	5·0849	

Line with slope 0·9121 through point 2·5568, 5·0849 is $y=0\cdot9121x+2\cdot7528$.

			3·6649	3·1091	6·1304	0·3065	3·4156	0·32404	3·2404	3·2404	11·06791	$[wxy]=16\cdot99595$
			4·5770	3·6548	2·7537	1·1015	4·7563	0·59602	5·9602	11·9204	28·34850	$[wx^2]=18\cdot5420$
			5·4891	6·3716	2·8263	0·8479	5·5237	0·58314	6·9977	20·9931	38·65320	$b=\dfrac{16\cdot99595}{18\cdot5420}$
			6·4012	6·9467	6·7832	0·6783	6·2684	0·29871	3·5845	14·3380	22·46908	
									19·7828	)50·4919	100·53869	$=0\cdot9166$
										2·5523	5·0821	

Line with slope 0·9166 through point 2·5523, 5·0821 is $y=0\cdot9166x+2\cdot7427$.

$$\begin{aligned}\chi^2&=[wy^2]-b[wxy]\\&=16\cdot04365-0\cdot9166(16\cdot99595)\\&=0\cdot46512\end{aligned}$$

$$V(b)=\frac{1}{[x^2]}=\frac{1}{18\cdot542}=0\cdot05395$$

$$\sigma_b=\sqrt{0\cdot05395}=0\cdot2323$$

$$x_m=\bar{x}-\frac{(\bar{y}-5)}{b}=2\cdot4672$$

$$\begin{aligned}s^2&=\frac{1}{b^2[nw]}+\frac{(5-\bar{y})^2}{b^4[wx^2]}=\frac{1}{b^2}\left\{\frac{1}{[nw]}+\frac{(5-\bar{y})^2}{b^2[wx^2]}\right\}\\&=1\cdot1905(0\cdot050544+0\cdot000433)\\&=0\cdot060881185\end{aligned}$$

and $s=0\cdot2463$

In the calculation in Table XV :

Column	
1	x=log. doses at intervals of 1·0 instead of intervals of 0·1761.
2	n=number of rats in each group.
3	P=proportion of rats giving positive responses.
4	Y=value of Y (probit) corresponding to each value of x, obtained from line drawn by inspection.
5	M.C.P.=maximum corrected probit for values >5, or minimum corrected probit for values <5, both obtained from Bliss' table.
6	Range, 1/Z, also obtained from Bliss' table.
7	P/Z=col. 3 × col. 6 for $Y>5$ or Q/Z=(1 − col. 3) × col. 6 for $Y<5$.
8	y=col. 5 + col. 7 for $Y<5$ or col. 5 − col. 7 for $Y>5$.
9	$w=Z^2/PQ$ obtained from Bliss' table.
10	nw=col. 2 × col. 9.
11	nwx=col. 1 × col. 10.
12	nwy=col. 8 × col. 10.

in Fisher and Yates *Statistical Tables for Biological, Agricultural and Medical Research.* The need for this weight factor is due to the fact that less credence can be placed on a percentage result approaching 0 or 100 than one nearer to 50%. After some argument as to how the weight factor should be applied it seems to be generally accepted now that the procedure should be thus. Plot the probit, *i.e.* 5+normal equivalent deviation (to avoid minus quantities) corresponding to the proportion of positive responses against the log. of each of the doses given, and draw, by inspection, the best straight line between them. Then measure or calculate the value of P which on this curve corresponds to the log. of each dose given, and calculate a fresh curve with weights based upon the values expected from the provisional computed line. If this is much different from the first curve, a third one is calculated with weights based upon the values obtained from the second. Sometimes even a fourth curve is calculated. This procedure will be most easily understood from the following example worked out from the imaginary result described in Chapter VII. For the purpose of this calculation, different numbers of rats have been assigned to the different groups, and, for simplifying the calculation, the values 1, 2, 3, 4

have been substituted for the logs. of the doses given, and the results at the end corrected by the factor, 0·1761, which is the log. of the ratio, 1·5, between successive doses.

When a value is required intermediate between any two values given in the tables, take a proportionate part of the difference between these two values.

The average, $\bar{x}$, is found by summing column 11 and dividing by the sum of column 10. Similarly $\bar{y}$ is found by summing column 12 and dividing by the sum of column 10. The curve of response is then computed from these figures by "least squares." The weighted squares and products of the deviations from the two means are determined as:

$$[wx^2]=S(wx^2)-\bar{x}S(wx)$$
$$[wxy]=S(wxy)-\bar{y}S(wx)$$
$$[wy^2]=S(wy^2)-\bar{y}S(wy)$$

Thus

$$[wx^2]=\left\{\begin{array}{r}3·020\\23·240\\64·854\\64·4928\end{array}\right\}-2·6103(52·3812)$$
$$=155·6068-136·7306$$
$$=\ 18·8762$$
$$[wxy]=S(wxy)-\bar{y}S(wx)$$
$$=\left\{\begin{array}{r}10·25290\\55·31120\\119·36379\\101·27788\end{array}\right\}-5·1337(52·3812)$$
$$=286·20577-268·90937$$
$$=\ 17·2964$$
$$b=\frac{[wxy]}{[wx^2]}=\frac{17·2964}{18·8762}=0·9164$$

The curve of response is then calculated from

$$\frac{y-y_1}{x-x_1}=b \text{ and } y=0·9164x+2·7416$$

The agreement between the observations and the line thus fitted is given by

$$\chi^2=[wy^2]-b[wxy]$$

$[wy^2]$ is calculated as the other deviations were and from it is subtracted $b[wxy]$. The difference, χ^2 was found to be 0·4705 which from the table of χ^2 entered in the line for $n=2$ (*i.e.* 4

observations less 2) shows it to be very small (1% point is 9·210), so small in fact that no further computation is really necessary. It has been made, however, to demonstrate the method.

Second computation.—The values of Y corresponding to the values 1, 2, 3, 4 for x in the equation $y=0·9164x+2·7416$ are calculated and entered in the fourth column of the table. The whole calculation is then repeated as in the first computation and, if desirable, a third computation is made, and even in some cases a fourth.

The values for χ^2, the variance of the slope, V(b), the standard deviation of the slope, σ_b, the value of the median fertility dose, x_m, with its variance s^2, and standard deviation, s, are obtained as shown.

The Potency of the Substance Tested.—Since the provisional units 1, 2, 3, 4 were used for the logs. of the lowest dose of the Standard and for each succeeding dose (which exceeded the one before it by 0·1761), the value for $\bar{x}_1$ must be transposed thus:

$$2·5523 \times 0·1761 - 0·2730$$
$$= 0·1765$$

$-0·2730 = \underset{\text{(log. 0·8mg.)}}{\bar{1}·9031} - 0·1761 =$ the distance between $x=0$ in the provisional units and $x=0$ in the true logs.

Since the provisional units 1, 2 were used for the logs. of the lower dose of test substance and for the higher dose (which exceeded that for the lower by 0·4771) the value for $\bar{x}_2$ must be transposed thus:

$$1·6890 \times 0·4771 - 0·7781$$
$$= 0·0277$$

$-0·7781 = \underset{\text{(log. 0·5g.)}}{\bar{1}·6990} - 0·4771 =$ the distance between $x=0$ in the provisional units and $x=0$ in the true logs.

Similarly, the slopes of the curve for the standard and test substance are corrected for the provisional doses thus:

$$b_1 = \frac{0·9166}{0·1761} = 5·2050 \text{ and } b_2 = \frac{1·4080}{0·4771} = 3·1608$$

The average slope is obtained by weighting each according to the total weighting factor (nw) of each curve ; thus

$$b = \frac{5·2050 \times 19·7828 + 3·1608 \times 11·0182}{19·7828 + 11·0182}$$
$$= 4·3763$$

TABLE XVI

TEST SUBSTANCE. 2 DOSES, 0·5 G. AND 1·5 G. IN RATIO 1 : 3. ∴ WORKING FACTOR IS 0·4771.

x	n	P	Y	Max Cd. Probit.	I/Z	P/Z	y	w	nw	nwx	nwy
1	10	0·1	3·7184	3·149	5·698	0·5698	3·7188	0·3427	3·427	3·427	12·74433
2	12	0·55	5·1257	6·263	2·528	1·1367	5·1263	0·6326	7·5912	15·1824	38·91479
									11·0182	)18·6094	51·65912
										1·6890	4·6885

$[wx^2]=2·3605$

$[wxy]=3·3237$

$b=\frac{3·3237}{2·3605}$

$=1·4080$

$$\chi^2=[wy^2]-b[wxy]$$
$$=4·6787-4·6797$$
$=0$ approximately (which, of course, was expected)

$$V(b)=\frac{1}{[x^2]}=\frac{1}{2·3605}=0·42364$$
$$\sigma_b=\sqrt{0·42364}=0·6508$$

$$x_m=\bar{x}-\frac{(\bar{y}-5)}{b}=1·6890+\frac{0·3115}{1·4080}$$
$$=1·6890+0·2212$$
$$=1·9102$$

$$s^2=\frac{1}{b^2[nw]}+\frac{(5-y)^2}{b^4[wx^2]}=\frac{1}{b^2}\left\{\frac{1}{[nw]}+\frac{(5-\bar{y})^2}{b^2[wx^2]}\right\}$$
$$=\frac{1}{1·9832}\left\{\frac{1}{11·0182}+\frac{0·3115}{1·9832\times 2·3605}\right\}$$
$$=\frac{1}{1·9832}(0·090759+0·06654)$$
$$=0·0793$$
and $s=0·2816$

The potency of the test substance is then found from the formula

$$x_1-x_2=\bar{x}_1-\bar{x}_2-\frac{(\bar{y}_1-\bar{y}_2)}{b}$$

$$=0\cdot1765-0\cdot0277-\frac{5\cdot0821-4\cdot6885}{4\cdot3763}$$

$$=0\cdot0589$$

$$=\log.\ 1\cdot1453$$

Therefore the potency of the test substance in grams is 1·1453 times the potency of the Standard in mg., *i.e.* 1g. of the test substance is equivalent to 1·1453mg. of the Standard, *i.e.* the test substance contains 1·1453 or, say, 1·15 International units of vitamin E per gram.

The Fiducial Limits of the Determination for P=0·95.—The fiducial limits are determined from the same formula as the one used when the response is measurable, *e.g.* increase in weight of the rat; but whereas, in that determination, the weight (w) attached to each average response was equal to n, the number of animals, in this determination the values of w are those obtained in the foregoing calculation. The limits have been worked out below in a form which has been found useful and economical of time in practice.

$$\bar{x}_1-\bar{x}_2-\frac{b(\bar{y}_1-\bar{y}_2)}{b^2-t^2\mathrm{B}}\pm\frac{t}{b^2-t^2\mathrm{B}}\{\mathrm{A}(b^2-t^2\mathrm{B})+\mathrm{B}(\bar{y}_1-\bar{y}_2)\}^{\frac{1}{2}}$$

This must be worked out with the logs. of the actual doses, not the 1, 2, 3, 4 and 1, 2 provisional logs., since the intervals in the two curves are different (0·1761 in the curve for the Standard and 0·4771 in the curve for the test substance).

$$\mathrm{A}=s^2\left\{\frac{1}{\mathrm{S}_1(w)}+\frac{1}{\mathrm{S}_2(w)}\right\}$$

$$=1\left\{\frac{1}{19\cdot7828}+\frac{1}{11\cdot0182}\right\}$$

$$=0\cdot1413$$

$$\mathrm{B}=s^2/\mathrm{S}_1\{w(x-\bar{x})^2\}+\mathrm{S}_2\{w(x-\bar{x})^2\}$$

$$=\frac{1}{3\cdot2747+1\cdot3142}$$

$$=0\cdot2179$$

$s^2=1$ in a quantal response.

$(x-\bar{x})$ for the two middle doses of Standard $=0{\cdot}5$

$(x-\bar{x})$ for the highest and lowest doses of Standard $=1{\cdot}5$

$$\therefore \quad S_1\{w(x-\bar{x})^2\}=(3{\cdot}2404+3{\cdot}5845)(1{\cdot}5)^2=15{\cdot}3560$$
$$+(5{\cdot}9602+6{\cdot}9977)(0{\cdot}5)^2=3{\cdot}2395$$
$$=18{\cdot}5955$$

Convert to actual logs. by $\times 0{\cdot}1761=\ 3{\cdot}2747$

$(x-\bar{x})$ for both doses of test substance $=\ 0{\cdot}5$

$$\therefore \quad S_2\{w(x-\bar{x})^2\}=(3{\cdot}4270+7{\cdot}5912)(0{\cdot}5)^2=\ 2{\cdot}7545$$

Convert to actual logs. by $\times 0{\cdot}4771=\ 1{\cdot}3142$

$$\begin{aligned}
(\text{St.})\ \bar{y}_1 &= 5{\cdot}0821\\
(\text{T})\ \bar{y}_2 &= 4{\cdot}6885\\
\bar{y}_1-\bar{y}_2 &= 0{\cdot}3936\\
b &= 4{\cdot}3763\\
b^2 &= 19{\cdot}1520\\
t &= 1{\cdot}96\ (P=0{\cdot}95)\\
t^2 &= 3{\cdot}8416\\
b^2-t^2B &= 19{\cdot}1520-0{\cdot}8371=18{\cdot}3149\\
b(\bar{y}_1-\bar{y}_2) &= 1{\cdot}7225\\
\frac{b(\bar{y}_1-\bar{y}_2)}{b^2-t^2B} &= 0{\cdot}09404\\
\frac{t}{b^2-t^2B} &= 0{\cdot}1070\\
A(b^2-t^2B) &= 2{\cdot}58789537\\
B(\bar{y}_1-\bar{y}_2)^2 &= 0{\cdot}03375271\\
\text{Total} &= 2{\cdot}62164808\\
\text{Square root} &= 1{\cdot}6192
\end{aligned}$$

Thus the limits are :

$$\bar{x}_1-\bar{x}_2-\frac{b(\bar{y}_1-\bar{y}_2)}{b^2-t^2B}\pm\frac{t}{b^2-t^2B}\{A(b^2-t^2B)+B(\bar{y}_1-\bar{y}_2)^2\}^{\frac{1}{2}}$$
$$=0{\cdot}1765-0{\cdot}0277-0{\cdot}0940\pm0{\cdot}1070\{1{\cdot}6192\}$$
$$=0{\cdot}0548\pm0{\cdot}1733$$
$$=+0{\cdot}2281 \text{ or } -0{\cdot}1185,\ \textit{i.e.}\ 0{\cdot}2281 \text{ or } \bar{1}{\cdot}8815$$
$$=\log.\ 1{\cdot}6908 \text{ or } \log.\ 0{\cdot}76578$$

Using the value $1{\cdot}1453$ (= antilog. of $0{\cdot}0589$, *not* of $0{\cdot}0548$) the limits are $147{\cdot}6$ to $66{\cdot}9\%$.

3. Analysis of Variance

Determinations are often arranged in such a way that known sources of error are eliminated, *e.g.* in a vitamin D determination, the rats of each litter are equally distributed amongst all the groups given the different doses of Standard and substance under test, for it has been shown that the variation in response to vitamin D is greater between litters (intralitter variation) than within a litter (interlitter variation). (Coward and Kassner, 1941.) Therefore, when the rats are so allocated to the various groups that all groups contain equal numbers of rats from each litter the error thus eliminated should be calculated and subtracted from the overall error of the test. It is done by a process called an analysis of variance thus :

The vitamin D content of the second U.S.P. reference cod liver oil was determined in the writer's laboratory by the "line-test" method. Three workers assessed the healing in each bone and the average of the three readings was taken as the healing for each bone. The results were collected in Table XVII, each column was totalled and averaged and each row likewise. Then the products of each column total and its average were added together, and likewise the products of each row and its average. The grand total, M, was multiplied by its mean, m, (Mm). This (Mm) was subtracted from each of the last totals, which gave the error due to differences between doses and the error due to differences between litters (*cf.* the procedure on page 176). The total error is obtained by squaring each observation, adding them together and subtracting Mm. Then the final analysis is made. The error due to random sampling is obtained as the total error less the errors due to differences between doses and that due to differences between litters. The number of degrees of freedom are 3 (*i.e.* 4–1) for doses, 14 (*i.e.* 15–1) for litters, and 60–1 (the total number of degrees of freedom) less those for doses and litters, *i.e.* 60–1–3–14=42 for random sampling. The mean square of the variance due to each factor is obtained by dividing each sum of squares by its degrees of freedom and the figure obtained for the variance due to random sampling (in this case 0·10966) is the value for s^2 to be use in estimating the accuracy of the test.

When s^2 of each group of this experiment was determined by method 2 described on page 176 and the average s^2 determined as described on page 184 it was found to be 0·1850, a much higher

TABLE XVII

An analysis of variance to obtain an estimate of the error due to random sampling when "litter-mate control" has been observed in the planning of the experiment

60 mg. C.L.O.	120 mg. C.L.O.	6 units vitamin D.	12 units vitamin D.	Totals.		Averages.	
1·333	1·167	1·0	1·333	A	4·833	a	1·20825
0·667	1·333	1·5	1·0	B	4·5	b	1·125
1·167	1·167	1·0	1·0	C	4·334	c	1·0835
0·5	1·833	0·667	1·0	D	4·0	d	1·0
1·0	1·333	0·833	0·5	E	3·666	e	0·9165
0·5	0·5	0·5	1·5	F	3·0	f	0·75
1·0	1·167	1·5	1·5	G	5·167	g	1·29175
1·333	1·667	1·5	2·167	H	6·667	h	1·66675
1·5	1·833	1·5	1·667	I	6·500	i	1·625
2·0	2·167	1·167	2·167	J	7·501	j	1·87525
1·667	2·0	1·5	1·667	K	6·834	k	1·7085
1·167	1·0	1·0	1·667	L	4·834	l	1·2085
1·0	2·5	1·0	1·667	M	6·167	m	1·54175
1·167	1·667	1·333	1·5	N	5·667	n	1·41675
1·0	1·833	0·833	1·5	O	5·166	o	1·2915
15)17·001 P	15)23·167 Q	15)16·833 R	15)21·835 S	M=78·836			19·70900
1·1334 p	1·5445 q	1·1222 r	1·4557 s			m=1·3140	

Pp	19·2689334
Qq	35·7814315
Rr	18·8899926
Ss	31·7852095
	105·7255670
Less Mm	103·59054
	2·13503

Aa	5·83947225
Bb	5·0625
Cc	4·6958890
Dd	4·0
Ee	3·3598890
Ff	2·25
Gg	6·67447225
Hh	11·11111111
Ii	10·5625
Jj	14·06625025
Kk	11·675889
Ll	5·841889
Mm	9·50797225
Nn	8·02872225
Oo	6·671889
	109·34844536
Less Mm	103·59054
	5·75791

Mm=103·59054

Sum of squares of observations=116·0891
Less Mm=103·59054
12·49856

	Sum of squares.	$d/f.$	Mean square.	Var. ratio.	0·1% points.
Error due to difference between doses ..	2·13503	3	0·71168	6·634	6·60 for $n_1/n_2=3/40$
Error due to difference between litters ..	5·75791	14	0·41128	3·834	3·64 for $n_1/n_2=12/40$
Error due to random sampling	4·60562	42	0·10966 $=s^2$		
	12·49856	59			

figure than the one obtained when the "litter-mate" control of the experiment was taken into account.

Further information from this analysis can be obtained by finding the ratio between the variance due to doses and that due to random sampling and also the ratio between the variance due to litters and that due to random sampling. Variance ratio tables are consulted (Tables V of Fisher and Yates' *Statistical Tables for Biometrical, Agricultural and Medical Research*) and entered in the appropriate column and row, according to the number of degrees of freedom for the numerator and denominator of the ratio. Fisher and Yates have four tables of Variance Ratio, 20% points not really significant, 5% points significant, 1% points very significant and 0·1% points highly significant. All four tables are consulted and that one noted in whose appropriate column and row occurs a figure most nearly equal to the variance ratio found in the experiment, *e.g.* the variance ratio of the errors due to differences in doses and random sampling was 6·634 with $n_1=3$ and $n_2=42$. There is no row for $n_2=42$ but there is a row for $n_2=40$, and one for $n_2=60$. In the 0·1% points table the variance ratio for $n_1/n_2=3/40$ is 6·60. For $n_1/n_2=3/42$ it would be slightly less (linear interpolation is allowable). Since the variance ratio 6·634 is even greater than 6·60, it indicates that the differences in healing due to differences in doses in this experiment are highly significant. Moreover, the variance ratio for errors due to litters and random sampling 3·834, where $n_1/n_2=14/24$ is greater than 3·64 in the 0·1% points table for $n_1/n_2=12/40$ and therefore the differences in the response of different litters must be regarded also as highly significant.

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CHAPTER XII

THE IMPROVEMENT OF BIOLOGICAL ASSAYS

1. The Influence of the Length of the Dosing Period on the Accuracy of a Vitamin Determination.
    (a) Vitamin A.
    (b) Vitamin B_1.
2. The Best Starting-point for the "Increase in Weight" Criterion.
3. Correlation between Increase in Weight during the Dosing Period of a Vitamin A Test and the Weight of the Rat when given the First Dose of Vitamin A.
4. Correlation between the Initial Weight of the Rat and the Amount of Healing brought about by a Dose of Vitamin D.
5. Co-variance between the Logarithm of the Weight of Ash and the Logarithm of the Weight of Organic Matter in the Determination of Vitamin D by the "Ash Content of the Bones" Method.
6. Interaction of Vitamins.
7. References.

WHEN a large number of biological assays have been made under the same conditions it is often possible to survey the data obtained with a view to discovering some modification in the technique by which the accuracy of the method might be increased, or the labour involved in the test diminished. A more or less casual survey will often suggest a point that can be examined fully by statistical methods. The following are typical of such investigations carried out in the writer's laboratory.

1. The Influence of the Length of the Dosing Period on the Accuracy of a Vitamin Determination

(a) *Vitamin A.*—It was felt for many years that the longer a vitamin A test was carried on, the greater was its accuracy. Many workers dosed their rats for 8 weeks; others felt that 5 weeks was the shortest reliable period. Coward (1933) examined the data obtained in drawing up her first curve of response to graded doses of vitamin A for 5 weeks and determined the slopes of the curves of response of the same rats for 1, 2, 3, and 4 weeks' periods respectively. The standard deviations of the increase in weight in 1, 2, 3, 4 and 5 weeks were determined from

these and from later data. Results from male and female rats were treated separately. About 1,000 male rats and about 1,200 female rats contributed information.

TABLE XVIII

THE INCREASE IN ACCURACY OBTAINED BY PROLONGING THE DURATION OF A VITAMIN A TEST FROM 1 TO 5 WEEKS

	s.	Curve of response.	Slope of curve.	$\lambda = \frac{s}{\text{slope}}$.
Male rats—				
1 week's test	6·82	$y = -0{\cdot}33 + 9{\cdot}05 \log. x$	9·05	0·75
2 weeks' test	9·68	$y = 3{\cdot}50 + 19{\cdot}28 \log. x$	19·28	0·50
3 weeks' test	11·86	$y = 6{\cdot}04 + 29{\cdot}62 \log. x$	29·62	0·40
4 weeks' test	13·70	$y = 10{\cdot}20 + 38{\cdot}07 \log. x$	38·07	0·36
5 weeks' test	15·32	$y = 13{\cdot}36 + 45{\cdot}81 \log. x$	45·81	0·33
Female rats—				
1 week's test	5·63	$y = 0{\cdot}94 + 6{\cdot}64 \log. x$	6·64	0·85
2 weeks' test	8·05	$y = 4{\cdot}10 + 13{\cdot}45 \log. x$	13·45	0·60
3 weeks' test	9·74	$y = 8{\cdot}24 + 17{\cdot}93 \log. x$	17·93	0·54
4 weeks' test	11·11	$y = 11{\cdot}43 + 22{\cdot}48 \log. x$	22·48	0·49
5 weeks' test	11·93	$y = 12{\cdot}55 + 27{\cdot}00 \log. x$	27·00	0·44

It may be seen in Table XVIII that the standard deviation of the increase in weight and slope of the curve of response both increase throughout the period of 1–5 weeks, but because the slope increases more rapidly than the standard deviation, $\lambda\left(=\frac{s}{\text{slope}}\right)$ which combines these two functions, decreases. Moreover, it decreases rapidly between the first and second weeks, less rapidly between the second and third, and less still between the third and fourth, and between the fourth and fifth. This applies to both male and female rats. The student would find it interesting to plot these results on graph paper. It was decided in this laboratory that the extra accuracy obtained by carrying on the test for 5 rather than for 3 weeks was usually not worth the extra labour involved.

It may be noted from Fig. 36, that at the end of one week (lowest curve), the male rats have responded to the lowest dose almost as well as to the highest, but that at the end of 5 weeks, the rats on the lowest dose have lost weight but those on the

higher doses have grown well. Similarly, the female rats do not show much discrimination between a low dose and a high dose in 1 week, but in 5 weeks on a low dose they maintain their weight and on higher doses they grow. If the 5 weeks' curves of male and female rats are superposed in one graph, they are seen to intercept each other at about the point $\bar{1}\cdot957$, *i.e.* at the point where the dose of cod liver oil was 0·90 (=antilog. $\bar{1}\cdot957$) or where the increase in weight for both males and females was about 11·4g. This means that the male rats respond less well than the female rats to low doses of vitamin A. Does this mean that the young male rat's need of vitamin A is greater than the young female's? A calculation of the mortality rates of young male and female

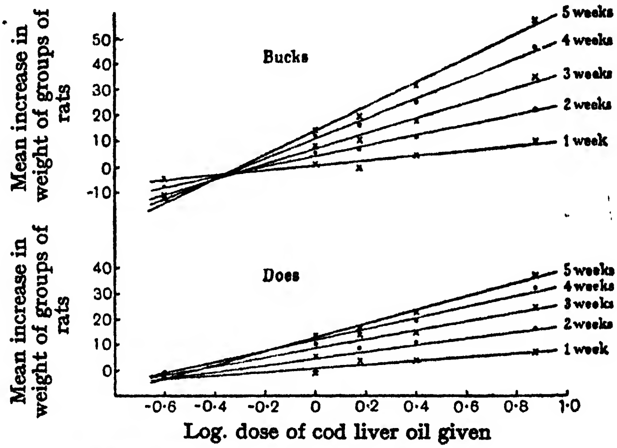

FIG. 36.—Curves of response relating increase in weight in 1, 2, 3, 4 and 5 weeks respectively to the logarithm of the dose of vitamin A given.

rats made by Gaddum and reported by Coward, Key, Dyer and Morgan (1931), and confirmed from much larger numbers of rats by Coward (1942) showed that the difference between the numbers of male and female rats dying on low doses of vitamin A was highly significant. A review of the mortality rates of human infants under one year of age for the years 1891 to 1938 showed that both had dropped enormously (from 164 to 60 per 1,000 births for males and from 133 to 46 for females), but that the ratio of rates for males to females had increased from 1·23 to 1·30. It was suggested that the greater (but still limited) amount of cod liver oil taken by pregnant mothers and by infants themselves may have been more effective on the female babies than

on the male, and thus have been a contributory cause to the rise in the *relative* mortality rates of males and females.

(*b*) *Vitamin B_1*.—After finding that the dosing period of a vitamin A determination could be reduced from 5 to 3 weeks without much loss of accuracy, Coward (1936) reviewed the results of vitamin B_1 determinations made in her laboratory since the introduction of the International Standard of Reference. They are summarised in Table XIX.

TABLE XIX

THE INCREASE IN ACCURACY OBTAINED BY PROLONGING THE DURATION OF A VITAMIN B_1 TEST FROM ONE TO THREE WEEKS

	s	b	$\lambda = \frac{s}{b}$
Male rats			
1 week's test	4·31	20·70	0·208
2 weeks' test	6·69	36·88	0·181
3 weeks' test	8·16	52·76	0·155
Female rats			
1 week's test	4·99	17·54	0·284
2 weeks' test	6·41	39·67	0·162
3 weeks' test	7·43	56·01	0·133

It is evident that, within the limits of time investigated, the longer the test is carried on, the less is the inaccuracy of the result, the female rats becoming more reliable than the male rats though starting from a lower point. If results from male and female rats are averaged, it will be seen that carrying on the test for 3 weeks does not make the test much more accurate than carrying it on for 2 weeks.

2. The Best Starting-point for the "Increase in Weight" Criterion

It was suggested at one time (Baumann *et al.*, 1934) that the period during which a rat regained the weight lost before being considered ready for dosing should be ignored and that the beginning of the increase in weight period should be the point at which the rat attained the highest weight it had previously reached in its preparatory period. This it would probably do,

if ever, within a fortnight of the beginning of dosing and Coward compared the accuracy, $\frac{s}{b}$, obtained in the first 3 weeks of a 5 weeks' test with that obtained in the last 3 weeks, basing the calculation on the figures used in the first calculation in this chapter. From Fig. 37 it may be seen that the variance in increase in weight for the male rats increased by nearly equal amounts each week for the 5 weeks, and for the female rats it also increased by equal amounts for the first 3 weeks but tailed off slightly in the fourth and again in the fifth week. The curve

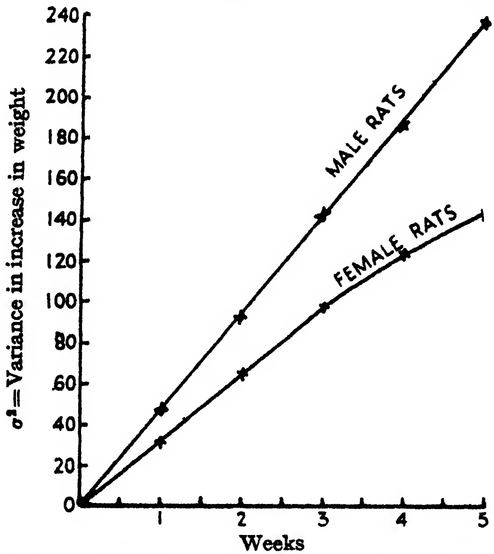

FIG. 37.—Variance in the increase in weight of male and female rats as the test is prolonged from 1 to 5 weeks.

of response for each separate week (*i.e.* the slope of each curve relative to the curve below it) was calculated from the curves for 1, 2, 3, 4 and 5 weeks' increases which had already been obtained (Table XVIII), by using the formula $\tan(\alpha-\beta) = \dfrac{\tan\alpha - \tan\beta}{1+\tan\alpha\tan\beta}$, where

e.g. $\tan\alpha$ was the slope (b_1) of the curve of response for a 2 weeks' test.

$\tan\beta$ was the slope (b_2) of the curve of response for a 1 week's test.

$\tan(\alpha-\beta)$ was the slope of the curve of response for the increase in weight during the second week.

The slopes thus obtained for the different weeks' increases in weight may be seen in Table XX. With the exception of the first week's slope for male rats, they fall into two nicely graded series.

TABLE XX

THE SLOPES OF THE CURVES OF RESPONSE RELATING INCREASE IN WEIGHT DURING THE FIRST, SECOND, THIRD, FOURTH AND FIFTH WEEKS RESPECTIVELY TO THE LOG. OF THE DOSE OF VITAMIN A GIVEN

Week.	Slope, *b*.	
	♂	♀
1	9·05	6·64
2	10·05	6·65
3	9·78	4·37
4	7·59	4·37
5	6·59	4·26

It is obvious that with the slope of each week's increase in weight steadily decreasing and the variance remaining nearly steady for the male rats and decreasing in the last weeks for the female rats, the inaccuracy of each week's response, as measured by $\frac{s}{b}$, is increasing week by week. Hence there can be no increase in accuracy in using the last 3 rather than the first 3 weeks' increases in weight, and this probably applies just as well to the procedure of counting the increase in weight from the point where the rat regained its highest weight of the preparatory period.

3. Correlation between Increase in Weight during the Dosing Period of a Vitamin A Test and the Weight of the Rat when given the First Dose of Vitamin A

It had often been suggested that the weight of a rat when given its first daily dose of vitamin A influenced the resultant increase in weight, and indeed, that the greater the weight of a rat the more vitamin A it required to bring about a particular increase. Cross (1945) put this to the test by plotting the increase in weight of rats during 3 weeks' dosing with 1 I.U. of vitamin A per day

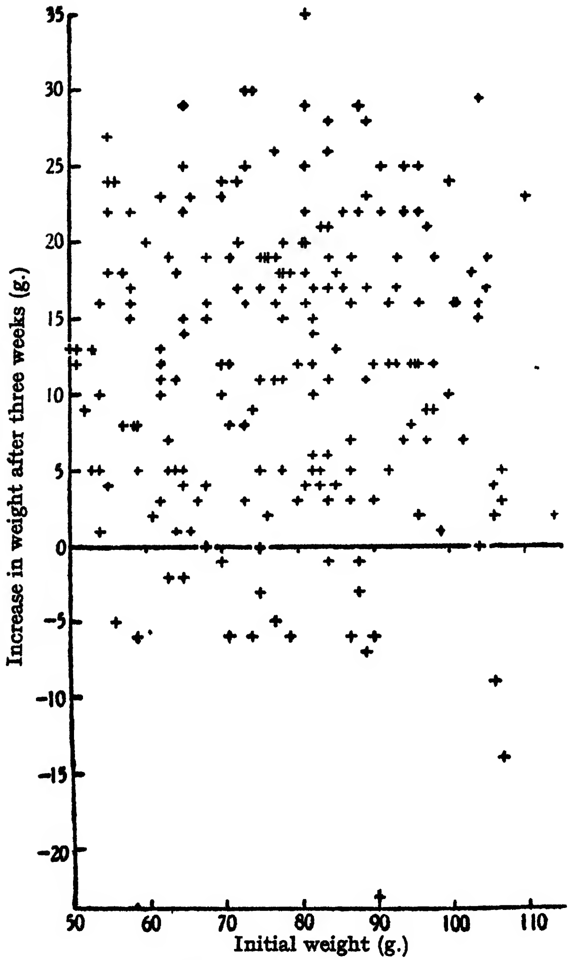

Fig. 38 (*a*).—200 males.

FIG. 38 (*a*) and (*b*).—Relation between the increase in weight of rats during 3 weeks' dosing with 1 I.U. of vitamin A (β-carotene) daily and the weight at which the rat was first given its dose (*i.e.* its weight when it had ceased to grow on a diet deficient in vitamin A).

against the weight when first given the dose (Fig. 38). This is called making a "dot diagram" and it often saves the extremely laborious process of working out the correlation coefficient. It did in this case for it is evident from the diagrams that there is no correlation between increase in weight and initial weight.

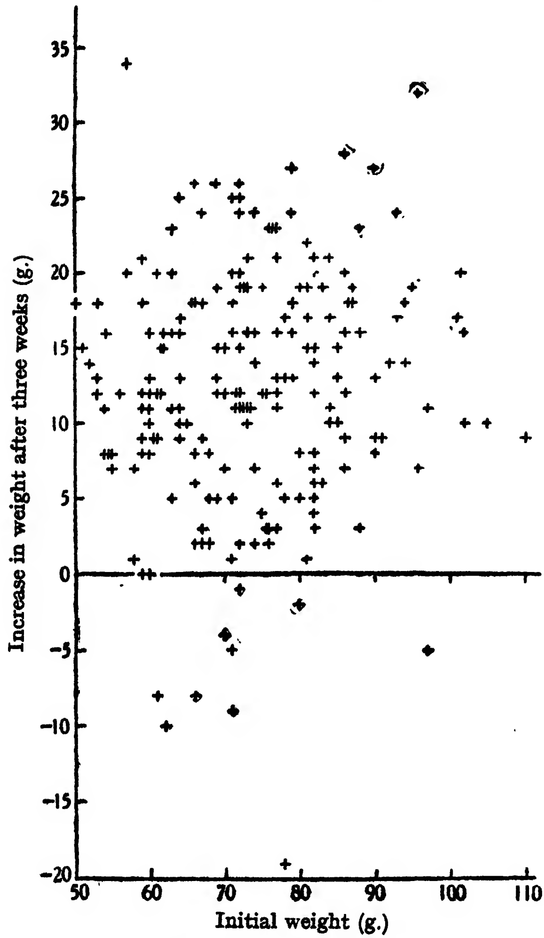

Fig. 38 (*b*).—200 females.

4. Correlation between the Initial Weight of the Rat and the Amount of Healing brought about by a Dose of Vitamin D

The initial weight here is the weight of the rat when it was first given the rachitogenic diet. Coward and Key (1933) worked this out by the formula

$$r = \frac{\Sigma(x-\bar{x})\,(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2 \,.\, \Sigma(y-\bar{y})^2}}$$

in which r=the correlation coefficient; $(x-\bar{x})$ is the difference

between each observation x and the mean, $\bar{x}$; $(y-\bar{y})$ is the difference between the corresponding value of y and the mean, $\bar{y}$. The results are collected in Table XXI.

TABLE XXI

CORRELATION BETWEEN THE INITIAL WEIGHT OF THE RAT (*i.e.* WHEN FIRST GIVEN THE RACHITOGENIC DIET) AND THE AMOUNT OF HEALING BROUGHT ABOUT BY A GIVEN DOSE OF VITAMIN D

Daily dose.	No. of rats.	Value of r.	P=probability that there is no correlation.
0·5 unit D	54 ♂	0·3388	slightly greater than 0·01
	41 ♀	0·2557	slightly less than 0·1
0·25 unit D	128 ♂	0·2286	slightly greater than 0·02 (for $n=100$)
	133 ♀	0·2780	slightly less than 0·01 (for $n=100$)

The probability that there was *no* correlation between the pairs of observations was found from the Table of r in Fisher's *Statistical Methods for Research Workers*, or in Fisher and Yates' *Statistical Tables for Biological, Agricultural and Medical Research*, which do not give values of r for $n>100$. The values for $n=54$ and for $n=41$ were found by plotting the necessary curves from the published tables, but a straight line connection between any two consecutive points could be accepted as accurate enough for interpolation. In this example, it is obvious that there was a strong correlation between the initial weight of the rat and the amount of healing produced by a particular dose of vitamin D. Ever since this was determined in the writer's laboratory, rats from the various litters have been assigned to the various groups according to initial weight, so that the total weights of the rats in the different groups have been as nearly equal as possible.

In the experiment by Coward and Kassner (1941) reported on page 205, the rats were thus assigned and by rearranging the data in fresh columns so that the rat of highest weight from each litter was in column 1, the next highest in column 2, and so on till the lightest rat was in column 6, the variance due to difference in initial weight was estimated and allowed for in the analysis of variance. It was only slight, however, as care had been taken to use litters of rats whose weights were as nearly uniform as the colony could provide.

5. Co-variance between the Logarithm of the Weight of Ash and the Logarithm of the Weight of Organic Matter in the Determination of Vitamin D by the "Ash Content of the Bones" Method

Bliss (1940) has subjected the figures given in this book (p. 133) to a very thorough statistical analysis. He contends that both the total amount of ash and the total amount of organic matter in the bone contribute information regarding the action of vitamin D. However, he first calculated the potency of the oil and the accuracy of the determination, using percentage ash as the criterion, by a method described by Bliss and Marks (1939) and found the potency of the oil to be $193 \pm 15{\cdot}0$ (st. error) units of vitamin D per gram (cf. 200 I.U. found by Coward from the diagram, Fig. 28). His calculation also indicated the validity of the assumption that the curves relating response to log. dose were linear and parallel.

Bliss then examined another assumption that had been made, viz. that the percentage of ash was constant at any given dose of vitamin D. In this calculation he also had to avoid loss in precision due to differences between litters. The percentage ash of each bone was replaced by the log. weight of the ash, which was adjusted by co-variance for differences in the log. weight of the organic matter. The analysis of variance then indicated that the amount of organic matter in the bones had but little effect upon their ash content, in this particular experiment, but it is easily conceivable that in some experiments it might have an effect. The potency of the oil measured thus (*i.e.* using log. weight of ash as criterion instead of percentage ash) was $197{\cdot}7 \pm 10{\cdot}6$ units per gram. The difference in potency from that obtained by using percentage ash was immaterial, but the estimate of the inaccuracy of the test was reduced from a standard error of $15{\cdot}0$ to $10{\cdot}6$, which would be equivalent to doubling the number of rats in a test whose result was calculated in the usual way.

$$\left[\frac{15^2}{10{\cdot}6^2} = \frac{225}{112{\cdot}36} = \frac{2}{1} \text{ (approx.)}\right]$$

From this it seems highly probable that most determinations of vitamin D by the "ash content of the bones" method may give a more accurate result when calculated from the log. weight of ash rather than from the percentage ash, and a more accurate estimate of the accuracy of this method would be obtained by

correcting the weight of ash by its observed relation to the weight of organic matter (in logarithms).

It was also shown that differences of sex in the rats was immaterial, and that the variance in response to a dose, whether measured as log. weights of ash or from values adjusted for differences both in organic matter and between litters, was independent of the mean response.

6. Interaction of Vitamins

One experiment is reported (Cross, Embleton and Coward) which can be examined for a possible interaction of vitamins. It is the one described in Chapter VIII under the heading "Interdependence of Vitamins." 80 rats given a diet free from vitamin B_1 and riboflavin, but complete in all other known essentials, were divided into 4 groups and given daily doses of :

Group	I	1 I.U.	of vitamin B_1	and	2·5μg.	riboflavin
,,	II	2 I.U.	,,	,,	2·5μg.	,,
,,	III	1 I.U.	,,	,,	5·0μg.	,,
,,	IV	2 I.U.	,,	,,	5·0μg.	,,

After 5 weeks the average increases in weight were :

Doses given.	1 I.U. vit. B_1.	2 I.U. vit. B_1.
2·5μg. riboflavin	24·85g.	29·25g.
5·0μg. ,,	29·70g.	35·40g.

An analysis of variance eliminating variance due to differences in dose and differences between litters (although this latter was not significantly greater than that due to random sampling) gave the variance of random sampling (s^2) as 41·35, whence $s=6{\cdot}43$.

(*a*) The slope of the curve for riboflavin when the diet contained 1 I.U. of vitamin B_1 was

$$\frac{29{\cdot}70-24{\cdot}85}{0{\cdot}3010}=16{\cdot}1$$

(*b*) The slope of the curve for riboflavin when the diet contained 2 I.U. of vitamin B_1 was

$$\frac{35{\cdot}40-29{\cdot}25}{0{\cdot}3010}=20{\cdot}4$$

(*c*) The slope of the curve for vitamin B_1 when the diet contained 2·5μg. of riboflavin was

$$\frac{29{\cdot}25-24{\cdot}85}{0{\cdot}3010}=14{\cdot}6$$

(*d*) The slope of the curve for vitamin B_1 when the diet contained 5·0μg. of riboflavin was

$$\frac{35{\cdot}40-29{\cdot}70}{0{\cdot}3010}=18{\cdot}9$$

The average error of any of these curves is given by :

$$\sigma_b^2=\frac{s^2}{Sw(x_1-\bar{x}_1)^2+Sw(x_2-\bar{x}_2)^2}$$

$$=\frac{41{\cdot}35}{40\times(0{\cdot}150)^2}$$

$$=\frac{41{\cdot}35}{40\times0{\cdot}0225}$$

$$=45{\cdot}94$$

and $\sigma_b=6{\cdot}78$

The significance of the difference between the slopes is given by the formula

$$\frac{M_1-M_2}{\sqrt{\epsilon_1{}^2+\Sigma_2{}^2}}=t$$

The value of t for slopes (*a*) and (*b*) is

$$\frac{20{\cdot}4-16{\cdot}1}{\sqrt{2\times45{\cdot}94}}=\frac{4{\cdot}3}{9{\cdot}585}=0{\cdot}4486 \text{ which is not significant.}$$

The value of t for slopes (*c*) and (*d*) is

$$\frac{18{\cdot}9-14{\cdot}6}{\sqrt{2\times45{\cdot}94}}=\frac{4{\cdot}3}{9{\cdot}585}=0{\cdot}4486 \text{ which is not significant.}$$

Thus neither of the slopes (for riboflavin or for vitamin B_1) has been increased by giving a larger dose of the other to all rats and hence it is concluded that vitamin B_1 and riboflavin have no interaction in the animal body.

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